REMARKS

Reconsideration of the application in light of the amendments and the following remarks is respectfully requested.

I. Status of the Claims

Upon entry of this amendment, claims 14, 23, 24, 33, 35 and 38-40 are pending in this application. Claims 39 and 40 have been added. Support for these claims is found throughout the specification, *e.g.*, at page 7, ll. 28-35, p. 8, ll. 14-20, p. 10, ll. 12-18, and pp. 28-29.

By this Amendment, no new matter has been added to the application.

II. Rejections Under 35 U.S.C. § 103(a)

A. The Examiner has rejected claims 14 and 24 as allegedly obvious over Saido *et al.*, *J. Biol. Chem.*, 269(21):15253-15257, 1994 ("Saido A") in view of Takeda Chem. Industries Ltd., EP 0 683 234 A1 ("Takeda"), Vigo-Pelfrey *et al.*, *J. Neurochem.*, 61:1965-1968, 1993 ("Vigo-Pelfrey") and Goding, Monoclonal Antibodies, Academic Press Inc., pp. 56-97 (1983) ("Goding").

The Examiner contends that Saido's polyclonal antibody 9204 is specific for the free N-terminus of Aβ and that it would have been obvious to modify Saido A's antibody to arrive at the antibody called for in claim 14 and 24 because Takeda teaches that antibodies specific for the N- and C-termini are useful for detection of Aβ species *in vitro*, because "Vigo-Pelfrey teaches that Aβ species are present in human CSF" and because of the general desirability of making monoclonal antibodies, such methods for making monoclonal antibodies being provided by Goding. The rejection is not believed to be well founded. First, Saido A fails to disclose an antibody that binds soluble Aβ. Second, there would have been no motivation to modify the antibody disclosed in Saido A to arrive at the antibody called for in claim.

1. The cited prior art fails to teach or suggest an antibody that binds soluble Aβ

Contrary to the Examiner's assertion, a monoclonal antibody with the features of polyclonal antibody 9204, does not meet all the limitations of claim 14. The mere fact that Aβ can exist in a soluble form in CSF, as taught by Vigo-Pelfrey, does not mean antibodies against Aβ will necessarily bind to soluble Aβ (*i.e.*, it may only detect an aggregated or otherwise insoluble form of Aβ). It is known for instance, that Aβ exists as aggregates and fibrils in CSF (*see* Exhibit 1-Pitschke *et al.*[1], *e.g.*, the abstract). Additionally, it is known that Aβ exists in different conformations, depending on whether it is soluble, non-fibrillar, and/or oligomeric, and therefore antibodies should be directed to these specific conformations. For example, U.S. Patent Application Publication 2005/0124016 (*see* Exhibit 2[2]) states at paragraph [0009]:

> In particular, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies proteolytically derived from the transmembrane amyloid precursor protein (APP) while not reacting with fibrillar amyloid β protein assemblies, monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies.

Moretto[3] also discloses antibodies that binds selectively to different Aβ conformers (See Exhibit 3). Specifically, Moretto states in the abstract:

> An ideal antigen should be soluble and nontoxic, avoid the C-terminally located T-cell epitope of Aβ, and yet be capable of eliciting antibodies that recognize Aβ fibrils and neurotoxic Aβ oligomers but not the physiological monomeric species of Aβ.

Additionally, exhibits 4[4] and 5[5] teach conformationally selective antibodies against Aβ.

Unlike the present invention (*see* p. 7, ll. 18-22, p. 8, ll. 14-20, p. 17, ll. 9-11, claims 1-13 as originally filed), Saido A is not concerned with therapeutic benefits of targeting Aβ, rather, Saido A is simply concerned with spatial resolution of the β-amyloidogenic process. Because of

[1] Pitschke *et al.*, *Nat Med.*, V. 4, pp. 832-834 (1998).
[2] Ladu *et al.*, U.S. Patent Application Publication No. 2005/0124016.
[3] Moretto *et al.*, *JBC*, V. 282, pp. 11436-11445 (2007).
[4] Glabe and Kayed, U.S. Patent Application Publication No. 2007/0110750.
[5] Lee *et al.*, *JBC*, V. 281, pp. 4292-4299 (2006).

this, it is of no consequence to Saido A whether specific forms of Aβ are detected, as Saido A is simply looking for the presence or absence of Aβ. Takeda, Vigo-Pelfrey and Goding do not cure this deficiency.

Applicants provide evidence, given in Exhibits 6 and 7, that antibodies detect soluble and insoluble forms of Aβ with different specificities. As can be seen by both ELISA and BIAcore analysis, a first antibody, "A", has a 10 fold higher affinity for the soluble form of Aβ (*see* Exhibit 6A), while the second antibody, "B", has a 100-fold higher affinity for the aggregated form of Aβ (*see* Exhibit 6B). Thus, antibodies that bind Aβ are not necessarily specific for the soluble form of Aβ, as compared to an insoluble or aggregated form of Aβ.

The discrepancy in binding affinity is not resolvable by the methods presented in Saido A. Furthermore, it is of no consequence to the questions Saido addresses, whether soluble or insoluble Aβ is targeted. The primary and secondary references cited by the Examiner do not show that Ab 9204 binds soluble Aβ. Saido A discloses *in vitro* experiments wherein Aβ is either fixed in section or denatured on a gel. Therefore, Saido A discloses antibodies that detect either insoluble Aβ (Fig. 3), or denatured Aβ (Fig. 1, 2 and 4). The form of Aβ detected by Saido A's antibodies is not the form called for in the present invention. Western blot and immunocytochemistry experiments cannot detect a specificity for binding soluble or insoluble Aβ, as neither uses soluble Aβ as a target. Therefore, Saido A's teachings fail to appreciate the difference between the insoluble and soluble forms of Aβ.

In fact, when probing for soluble and insoluble Aβ in a western blot experiment, the binding preference of antibodies, known to have a preference, cannot be ascertained (*see* Exhibit 7-western blot). Antibody B fails to show the preference for aggregated Aβ, as it detects both soluble and aggregated forms of Aβ.

There is no doubt that Aβ bound to nitrocellulose or present in fixed sections is not Aβ soluble in CSF. Moreover, in view of the denatured and fixed and/or bound state of the Aβ that is present in the Western blotting experiments and in the sections, there is no expectation that the Aβ

recognized in these experiments has a structure similar to Aβ that is soluble in CSF. Thus, an observation that antibody 9204 binds to Aβ in Western blotting experiments and in fixed sections does not support a conclusion that antibody 9204 binds to Aβ that is soluble in CSF. If Aβ is denatured on a gel, a preference for the soluble form of Aβ over the insoluble form of Aβ cannot be ascertained.

2. The prior art provides no motivation to arrive at an antibody that binds soluble Aβ

The Examiner's obviousness argument is based on the contention that since it is known Aβ is soluble in CSF (Vigo-Pelfrey), it follows one would have been motivated from the teachings of Saido A, Takeda and Goding to generate monoclonal antibodies specific for the soluble form of Aβ. The Examiner fails to appreciate that an antibody can be specific for different conformers of Aβ, *i.e.*, a soluble or insoluble conformation.

Saido A does not provide any suggestion to obtain or choose an antibody that is specific for Aβ that is soluble in CSF. Saido A is directed entirely to using antibodies as diagnostic tools, either *in vitro* as probes in Western blotting, or in the staining of tissue sections, wherein Aβ peptides are fixed to a solid substrate. Saido A is not concerned with therapeutic methods that specifically target one form of Aβ. Thus, Saido A provides no hint or suggestion that there would have been any benefit to obtaining a free end specific antibody that binds Aβ that is soluble in CSF, as called for in claim 14. The Examiner does not cite a teaching as to how one of ordinary skill in the art would modify antibody 9204 to make a monoclonal antibody specific for the soluble form of Aβ. Therefore, one of ordinary skill in the art would not have been motivated from the teachings of Saido A to generate such an antibody.

The antibody in Saido A, which binds to a denatured or insoluble form of Aβ, does not necessarily bind to the soluble form of Aβ. Vigo-Pelfrey does not provide any teachings directed to targeting soluble Aβ. The fact that Aβ is soluble in CSF does not mean an antibody will bind soluble Aβ. Moreover, none of the references cited by the Examiner provide a teaching that it

would be useful to target soluble Aβ over insoluble Aβ. Saido and Takeda are concerned with detection of Aβ, in general, and not the specific forms. Moreover, in each case, the antibody is useful for the questions asked. Therefore, upon a fair reading of Saido and Takeda, one of ordinary skill in the art would not have been motivated to generate other Aβ antibodies, as the ones given were sufficient for their purposes.

Takeda does not provide suggestion or motivation that would have led those skilled in the art to modify antibody 9204 such that it binds Aβ that is soluble in CSF. The Examiner states that Takeda teaches that certain monoclonal antibodies are specific for the N- and C-termini of Aβ are useful for the detection of Aβ 1-40 and Aβ 1-42 for the detection of Aβ species *in vitro* and that Takeda teaches that Aβ1-40 is water soluble. There is no nexus, however, between the fact that Aβ 1-40 may be water soluble and the use of antibodies as diagnostic tools *in vitro* that would suggest modifying antibody 9204 to arrive at an antibody that binds Aβ that is soluble in CSF, as called for in claim 14. As set forth above, Saido A demonstrates that Aβ antibodies that bind Aβ bound to nitrocellulose and in fixed sections are perfectly suitable for use as diagnostic tools. Moreover Saido A does not disclose or suggest that his antibodies would be useful for therapy purposes, contrary to the present invention. In short, Takeda's disclosure that Aβ 1-40 is soluble adds nothing to Saido A, because neither Takeda nor Saido A, nor any knowledge common to those of ordinary skill provides any suggestion or appreciation that it would be desirable to modify antibody 9204 such that it binds to soluble Aβ.

Vigo-Pelfrey does not cure the deficiencies of Saido A, either alone or in combination with Takeda and Goding. Vigo-Pelfrey teaches Aβ can be soluble in CSF. Applicants do not dispute this. In fact, targeting the soluble form of Aβ is the basis of the Applicants' invention. However, the mere fact that Aβ is soluble in CSF does not mean that all antibodies grown against Aβ or portions thereof will detect the soluble form, for reasons given above.

Nor does Goding cure the defects in Saido A, either alone or in combination with Takeda and Vigo-Pelfrey. The Examiner cites Goding as teaching routine methods of making monoclonal antibodies and for the motivation to make monoclonal antibodies to decrease the lot to lot variability

that can result with polyclonal antisera. The Examiner cites nothing in Goding that would suggest modifying the polyclonal antiserum disclosed in Saido A such that it is free-end specific for Aβ that is soluble in CSF.

For at least these reasons, claims 14 and 24 are not obvious over Saido A, Takeda, Vigo-Pelfrey and Goding.

B. Claims 14 and 33 are rejected under 35 U.S.C. § 103(a) as being allegedly obvious over Takeda in view of Saido A, Saido B, Vigo-Pelfrey and Goding. The rejection is not believed to be well taken, as follows.

As a starting point for the instant rejection, the Examiner cites Takeda's monoclonal antibody BA-27a. The Examiner concedes that BA-27a does not have the specificity called for in claim 14 or 33. As the Examiner acknowledges, BA-27a binds to Aβ1-38 and Aβ-39. Thus, BA-27a binds to Aβ species that are at least two amino acids shorter than Aβ1-40. Accordingly, BA-27a is not "specific for the C-terminus of Aβ-40," as called for in claim 14. As the Examiner further acknowledges, BA-27a also binds to Aβ1-42. Thus, there is no evidence that BA-27a has the property that it will not bind APP.

The Examiner attempts to cure the defects in Takeda by citing Saido A, Saido B, Vigo-Pelfrey and Goding, but the attempt is not well taken, for the following reason. First, there is no suggestion in Saido A and Saido B or in the state of the art generally to modify any procedure in Takeda to produce to antibodies that are free-end specific for the free-end of Aβ1-40 but do not bind APP. The Examiner correctly cites Saido A for teaching that "similar approaches [to those in Saido A] for producing the proteolytic product specific antibodies will be applicable to resolving the differential carboxy-terminal of Aβ peptides" and that Saido's "unique methodology" seems to have general applicability. The Examiner, however, fails to correctly identify the Saido's "unique methodology" that would yield a "similar approach." Thus, the "unique approach" set forth in both Saido A and Saido B is a two-step approach wherein an immunization is performed with peptide conjugated to KLH to obtain a polyclonal antiserum, which is then affinity purified against peptide

immobilized to Affi-Gel 10. Saido A at page 15253, "Experimental Procedures" section. Thus, the Examiner is mistaken to conclude that it would have been obvious to use the "conventional end-peptide immunization techniques of Saido A and B" with monoclonal antibody technology, because upon reading Saido A and B one of ordinary skill in the art would recognize it as the two-step approach set forth in Saido A that leads to the antibody with the desired specificity. Saido's two-step procedure is incompatible with production of monoclonal antibodies, as disclosed in Takeda. Thus, for this reason there is no suggestion to combine Takeda with Saido A, Saido B and Goding to arrive the antibody called for in claim 14.

C. Claims 23 and 35 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Saido A, Takeda, Vigo-Pelfrey and Goding as applied to claim 14 and 24 above and further in view of U.S. Patent No. 6,114,133 to Seubert ("Seubert") and Bio Techniques, 16(3):476-483, 1994 to Duenas et al. ("Duenas").

As set forth above, claim 14 is not obvious over the combination of Saido, Takeda, Vigo-Pelfrey and Goding for at least the reason that these documents do not suggest or teach a monoclonal antibody that binds specifically to a free amino terminal end of Aβ that is soluble in CSF. Claims 23 and 35 call for the same binding characteristics as the antibody in claim 14, but differ from claim 14 in that they call for a single chain antibody. Thus, for at least the same reasons as claim 14, claims 23 and 35 are not obvious over the combination Saido, Takeda, and Goding. The Examiner cites Seubert and Duenas simply for single chain antibodies, as called for in claim 23 and 35. Thus, neither Seubert nor Duenas, separately or in combination, provides information required to cure the defects in Saido, Takeda, Vigo-Pelfrey and Goding. Thus, claims 23 and 35 are not obvious over any combination of Saido, Takeda, Goding, Vigo-Pelfrey, Seubert and Duenas. Reconsideration of the claims and withdrawal of the instant rejection is requested.

D. Claims 23 and 38 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Takeda, SaidoA, Saido B, Vigo-Pelfrey and Goding as applied to claim 14 and 33 above and further in view of Seubert and Duenas.

The Examiner further rejects claims 23 and 38 over the combination of Takeda in view of Saido A, Saido B, and Goding and further in view of Seubert and Duenas. The rejection is traversed. The Examiner cites Seubert and Duenas solely for their teaching of single chain antibodies. Accordingly, no combination of Seubert and Duenas cures the defects in the combination of Takeda with Saido A, Saido B, Vigo-Pelfrey and Goding that is set forth immediately above. Accordingly, claims 23 and 33 are not obvious over any combination of Takeda in view of Saido A, Saido B, and Goding and further in view of Seubert and Duenas.

For at least the reasons set forth above, claims 14, 23 and 33 are not obvious over Takeda in view of Saido A, Saido B, and Goding and further in view of Seubert and Duenas. Reconsideration of the claims and withdrawal of the instant rejection is requested.

III. New Claims 39 and 40

New claims 39 and 40 are directed to free end specific Aβ neutralizing antibodies that bind Aβ that is soluble in CSF and inhibit Aβ neurotoxicity. Claims 39 and 40 are patentable over the prior art of record. Saido A is concerned with diagnostic methods to probe for the presence of Aβ. Saido A does not disclose an antibody that binds soluble Aβ and inhibits neurotoxicity. Nor does Takeda suggest such an antibody. Nor do Saido B, Vigo-Pelfrey, Goding, Seubert and Duenas separately or in combination disclose or suggest such an antibody. Saido B, Goding, Seubert and Duenas are directed to methods of making antibodies, and are not concerned with antibodies that bind soluble Aβ to inhibit neurotoxicity of Aβ, as called for in claims 39 and 40. Similarly, Vigo-Pelfrey is not concerned with inhibiting neurotoxicity, and thus does not suggest the antibodies of claims 39 and 40.

For at least these reasons, claims 39 and 40 are patentable over the prior art of record. Additionally, claims 39 and 40 are believed to comply with §101 and §112.

Allowance of claims 39 and 40 is respectfully requested.

IV. Conclusion

This application is believed to be in condition for allowance which is earnestly solicited. If the Examiner believes there are outstanding issues that could be advanced by an Examiner's interview or an Examiner's amendment, the Examiner is invited to contact Applicant's attorney listed below.

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List of Exhibits

1. Pitschke *et al.*, *Nat Med.*, V. 4, pp. 832-834 (1998).
2. Ladu *et al.*, U.S. Patent Application Publication No. 2005/0124016.
3. Moretto *et al.*, *JBC*, V. 282, pp. 11436-11445 (2007).
4. Glabe and Kayed, U.S. Patent Application Publication No. 2007/0110750.
5. Lee *et al.*, *JBC*, V. 281, pp. 4292-4299 (2006).
6. Aβ selective antibody data.
7. Western Blot data.

Exhibit 1

In connection with Application No. 09/402,820

Detection of single amyloid β-protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy

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Alzheimer's disease is associated with the intraparenchymal growth of plaque-like amyloid deposits[1-3]. Amyloid plaques are formed by the progressive deposition and transformation of soluble amyloid β-protein monomers into insoluble and fibrillar aggregates that contain amyloid β-protein in a β-pleated sheet conformation. This process is described as 'seeded polymerization' of the monomers with slow-nucleation and fast-growth kinetics[4]. Soluble amyloid β-protein monomers are present in the cortical extracellular space and in the cerebrospinal fluid[5,6], whereas insoluble aggregates so far can be found only by the examination of brain tissue by biopsy or autopsy. Here we present a biophysical method that uses the principle of seeded polymerization in combination with fluorescence correlation spectroscopy, which allowed us to detect single amyloid β-peptide aggregates in the cerebrospinal fluid samples from Alzheimer's patients. All of 15 Alzheimer's samples but none of the 19 age-matched control samples produced large peaks with fluorescence correlation spectroscopy indicating the rapid aggregation of the fluorescent labelled synthetic amyloid β-protein probe onto the amyloid β-protein 'seeds' present in the cerebrospinal fluid. Our method could enable easy *in vivo* detection of the cerebral amyloid β-protein pathology of Alzheimer's disease and might be of potential value to facilitate its routine diagnosis.

Because Alzheimer's disease (AD) resembles prion diseases in pathogenic protein multimerization[7] and we have studied the mechanism of prion protein (PrP) multimerization using fluorescence correlation spectroscopy (FCS) (K. Post *et al.*, manuscript submitted), we used FCS to study amyloid β-protein (Aβ)–multimerization. In FCS, fluorescence-labeled molecules are analyzed in a volume of less than a femtoliter, which is delimited by confocal illumination with a laser and image formation on a small aperture in front of the fluorescence detector[8]. From the autocorrelation function of the fluorescence intensity, the diffusion time of the molecules through the confocal volume is measured and from that the molecular weight can be calculated. Thus, the method is very powerful for studying aggregation processes.

Synthetic amyloid β-peptides in aqueous buffers tend to self-aggregate[9,10]. Here, we analyzed this process quantitatively by FCS after labeling the Aβ with a fluorescent marker. While many but fairly small aggregates are in solution, a fluctuating fluorescence signal is seen, which represents fluctuations of the number of fluorophores in the confocal volume (Fig. 1*a*). Depending on the concentration of the Aβ and the incubation time (in our experiments, after about 30 minutes), a portion of the amyloid β-peptides are sometimes multimerized into larger aggregates that are detected by FCS as larger fluctuations (data not shown). The first steps in aggregate formation of Aβ can be detected at concentrations as low as 100 nM using the sensitive method of FCS for detection, whereas the use of more conventional techniques like light scattering and sedimentation allow detection of insoluble aggregates only in the micromolar concentration range[11].

We studied seeded multimerization, a much faster growth process than spontaneous multimerization, by adding labelled amyloid β-peptides to pre-existing multimeric particles. We tested whether Aβ multimers in the cerebrospinal fluid (CSF) of AD patients might act as 'seeds' for polymerization. In our study of the PrP, spontaneous multimerization was initiated when the concentration of SDS in the solution was lowered from 0.2% (which kept PrP soluble) to below 0.02%. Thus, we added fluorescence-labeled Aβ1–42, which was kept soluble in aqueous buffer containing 0.2% SDS, to human CSF samples, thereby diluting it to 0.02% SDS. The final Aβ concentration was less than 100 nM, far below the critical solubility limit[4]. Samples from the non-AD control group produced a fluctuating signal in the first 20 minutes after addition of the Aβ (Fig. 1*a*); however, those from AD-patients produced high-intensity peaks (Fig. 1*b*). Rarely, bursts were also produced by samples from the control group, which might be caused by sponta-

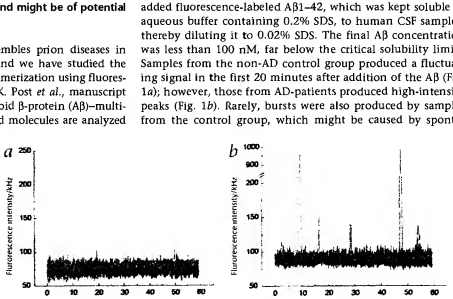

Fig. 1 Time course of the fluorescence intensity in FCS after the addition of fluorescence-labeled Aβ1–42 to control (***a***) and AD (***b***) CSF. Each large peak in *b* represents a single multimeric particle to which labelled Aβ1–42 is bound. Twenty scans of 60 seconds' observation time each are recorded in series and superimposed. AD-specific peaks are more than three times the average fluctuation of the fluorescence intensity.

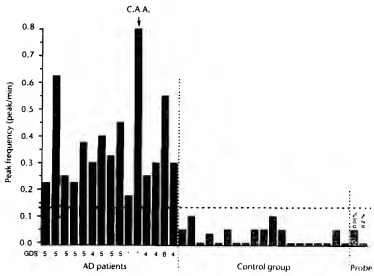

Fig. 2 Selective detection of labeled Aβ1–42 deposited on multimeric particles in the CSF of AD patients and controls. 'Probe' measurements represent the background from labeled Aβ1–42 in phosphate buffer, either in 0.2% SDS or after dilution to 0.01% SDS, with a few events of spontaneous multimerization. C.A.A., the patient with cerebral amyloid angiopathy; GDS, Global Deterioration Scale; *, GDS not done.

neous multimerization of the fluorescent Aβ during the time span of the experiment (about 30 min) or by deposition of Aβ on other structures (for example, cell fragments) in the CSF. The number of peaks counted in 20–30 minutes in samples from patients (n = 15) is more than those from the control group (n = 19), and no overlap was detected between these groups (Fig. 2). A patient with cerebral amyloid angiopathy (a condition closely related to AD, with marked vascular Aβ deposition) produced the highest peak frequency. Comparing peak frequencies measured in the control group with those from spontaneous multimerization of the Aβ probe in phosphate buffer indicates that most of the unspecific peaks in these analyses might originate from spontaneous multimerization of the probe.

To analyze the nature of the particles detected in the CSF of AD patients, we compared immunoprecipitation with the monoclonal Aβ antibody 6E10 with that of an unrelated antibody (Fig. 3*a*). High concentrations of antibody 6E10 (that is, a 250-fold dilution of ascites fluid) reduced the peak frequency in AD CSF. Neither lower concentrations (that is, a 500-fold or higher dilution) nor the addition of the unrelated antibody produced a significant reduction of the peak frequency. Addition of 1 mM Congo Red, which is known to inhibit the fibrillogenesis of Aβ (ref. 12), also lowered the peak frequency of Aβ-incorporation; addition of Congo Red to pre-formed synthetic Aβ1–42 peptide multimers or to the CSF of AD patients produced peak frequencies 12–40% that of samples without Congo Red (Fig. 3*b*). In the presence of increased metal ion concentrations, the aggregation phenomenon of Aβ particles might be shifted to lower concentrations or faster aggregation, as it favors Aβ fibrillogenesis[13,14]. The addition of 1mM $ZnCl_2$ resulted in a higher number of peaks (Fig. 3*c*). However, 1 mM $ZnCl_2$ also produced increased spontaneous multimerization of the Aβ probe, indicated by the results with control CSF. Therefore, the additional effect of Zn^{2+} on Aβ incorporation was difficult to interpret quantitatively, but seemed significant in two AD CSF samples. Thus we conclude from three independent lines of evidence that the particles detected in CSF of AD patients are mostly multimeric amyloid β-proteins.

Immunoprecipitation allowed us to deplete the CSF of amyloid β-proteins, but the amount of the precipitated Aβ was too small for additional biochemical characterization. Gel electrophoresis after immunoprecipitation of Aβ-aggregates formed *in vivo* has been reported, but was restricted to oligomeric Aβ-aggregates produced in cell-cultures[15].

To optimize the detection of Aβ multimers in CSF, the peak frequencies after the addition of Aβ1–42 or Aβ1–40 to CSF samples were compared and analyzed for their dependence on the SDS concentration after dilution. Aβ1–42 produced significantly higher peak frequencies when used as a probe for AD-specific particles in the CSF, and an SDS concentration of 0.02% was the best compromise between sensitivity and high specifity, that is, suppression of spontaneous multimerization (data not shown).

The linearity of the FCS-determined peak frequency with the concentration of AD-specific particles could not be tested with CSF samples from AD patients because the number of those par-

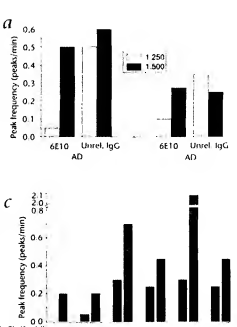

Fig. 3 Analysis of the type of particles detected in the CSF of AD patients. ***a***, Decrease in peak frequency after immunoprecipitation with monoclonal anti-Aβ antibody 6E10 compared with immunoprecipitation with an unrelated monoclonal antibody. ***b***, Decrease in peak frequency with the addition of Congo Red (CR). The CSF of three AD patients as well as synthetic pre-formed Aβ1–42 multimers were analyzed in the presence (+) and absence (-) of 1 mM Congo Red. ***c***, Increase in peak frequency with the addition of 1mM $ZnCl_2$. The CSF of four AD patients and one control patient as well as the Aβ probe in buffer were analyzed. AD(*), samples with a significant additional effect from $ZnCl_2$.

ticles could not be determined independently. Therefore, the linearity was tested with synthetic Aβ multimers and could be verified over almost two orders of magnitude (20 ng to 1000 ng multimer per 20μl sample volume) with an average deviation of about 15% (data not shown).

Here, using FCS to study the multimerization of the Aβ peptide allowed us to differentiate between spontaneous and seeded multimerization. However, seeded multimerization was exploited in a specific situation: In the CSF, very large multimers of Aβ are present, to which monomeric fluorescent amyloid β-peptides are added. Additional results indicated that the major component of the particles detected in the CSF of AD patients was Aβ multimers. We demonstrated that large Aβ multimers could be detected in the CSF only from AD patients, whereas it is known that the amount of soluble Aβ in CSF of AD patients is either unchanged or decreased[16,17]; a decrease of soluble Aβ might result from Aβ multimer formation. Therefore, the specific detection of Aβ multimers in the CSF could be a diagnostic test for AD. Here we concentrated on the biophysical basics of this new method; more extensive clinical studies will detail its sensitivity and specificity as a diagnostic test and demonstrate the relation between FCS-determined peak frequency and the clinical and pathological stage of AD. The inclusion of preclinical familial AD cases will demonstrate its validity in testing for early AD. Moreover, our study began with prion research and the principle of seeded multimerization could be extended to prion diseases.

Methods

Cerebrospinal fluid from Alzheimer patients and controls. CSF was obtained from 14 clinically diagnosed AD patients (mean age, 74.1 years; range 56–88 years) and one 66-year-old patient with the clinical diagnosis of cerebral amyloid angiopathy. All AD patients were diagnosed according to the ICD 10 criteria[18] after medical, neurological and psychiatric histories and examinations had been obtained, including electroencephalography and computerized brain tomography. The mean score of the Global Deterioration Scale[19] was 4.8. Control CSF samples were obtained from 19 neurological patients (mean age, 67.1 years; range 51–87 years) with diagnoses such as multi-infarct dementia, cerebral infarction, epilepsy and peripheral neuropathy, who had normal results by routine CSF examination. All samples were obtained by lumbar puncture, frozen within 2 h and stored at –70 °C before analysis; repeated freeze/thaw cycles were avoided.

Fluorescence correlation spectroscopy (FCS). We used a ConfoCor instrument from Evotec Biosystems GmbH (Hamburg, Germany) and Zeiss Jena GmbH (Germany). Measurements were taken in chambered tissue culture cover glasses with borosilicate bottoms [AUTHOR: OK?] (Nalge-Nunc, Naperville, Illinois). FCS was done immediatly after 18 μl of CSF were added to 2 μl of labelled peptide (4 ng/μl Aβ1–42 in 10 mM sodium phosphate, pH 7.2, 0.2% SDS).

Fluorescence labeling of amyloid β-peptides. Aβ1–42 and Aβ1–40 were purchased from Bachem Biochemica (Heidelberg, Germany). They were solubilized in a stock solution of 400 μM in water-free DMSO and stored at –70 °C. The peptides were labeled at their primary amino groups with the fluorophor Cy2 (Amersham), which had the smallest influence on the self-association and seeded multimerization reaction (K. Post *et al.*, manuscript submitted). For labeling, 18 μg of amyloid β-proteins were incubated at 4 °C for 1 h with 100 μg Cy2, in a total volume of 120 μl (50 mM sodium phosphate, pH 7.2 containing 0.2% SDS). Degradation of the peptides was inhibited by addition of 7.5 mM PMSF. The Aβ–Cy2 conjugates were separated from free Cy2 by a gel filtration spin column (MoBiTec, Göttingen, Germany) and by gel filtration with a NAP5-column (Pharmacia). The best results were obtained by the addition of 0.2 M NaCl and 0.2% SDS to the elution buffer (10 mM sodium phosphate, pH 7.2).

Immunoprecipitation. Ascites fluid containing the 6E10 Aβ antibody (Senetek, Maryland Heights, Missouri) or an unrelated antibody against calmodulin (Sigma) was added to 100 μl of CSF at a given dilution and incubated for 16 h at 4 °C. After incubation with Protein A-conjugated sepharose (15 μg/μl; Pharmacia) for 2 hours and centrifugation (12,000*g* for 5 min), labeled Aβ was added to the supernatant, and FCS measurements were taken.

Aβ multimers. Synthetic preformed Aβ multimers were obtained by twentyfold dilution of 400 μM Aβ in DMSO in phosphate buffer and incubation for 16 h (Aβ1–42) or 72 h (Aβ1–40).

Acknowledgements

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Exhibit 2

In connection with Application No. 09/402,820

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(54) **ANTIBODIES SPECIFIC FOR TOXIC AMYLOID BETA PROTEIN OLIGOMERS**

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(57) **ABSTRACT**

The present invention provides compositions and methods for diagnosing Alzheimer's disease (AD). In particular, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies proteolytically derived from the transmembrane amyloid precursor protein (APP) while not reacting with fibrillar amyloid β protein assemblies, monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies, and methods of use of these compositions in the diagnosis of Alzheimer's disease, as well as methods to monitor treatment and/or disease progression of AD in patients.

FIGURE 1

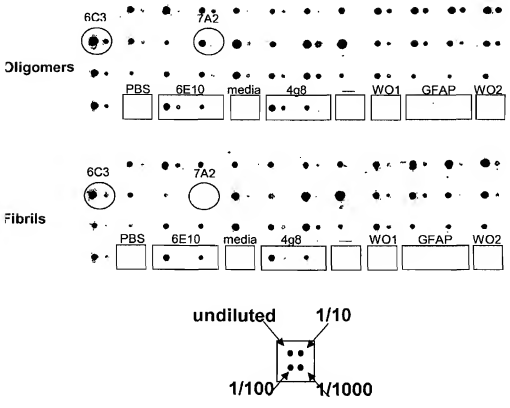

6C3
7A2
Oligomers
PBS
6E10
media
4g8
—
WO1
GFAP
WO2
6C3
7A2
Fibrils
PBS
6E10
media
4g8
—
WO1
GFAP
WO2
undiluted
1/10
1/100
1/1000

FIGURE 2

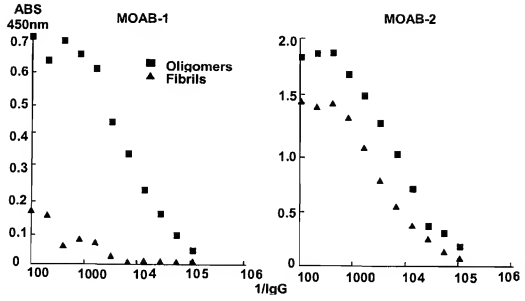

ABS
450nm
MOAB-1
Oligomers
Fibrils
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
1000
104
105
106
1/IgG
MOAB-2
2.0
1.5
1.0
0.5
0
100
1000
104
105
106

FIGURE 3

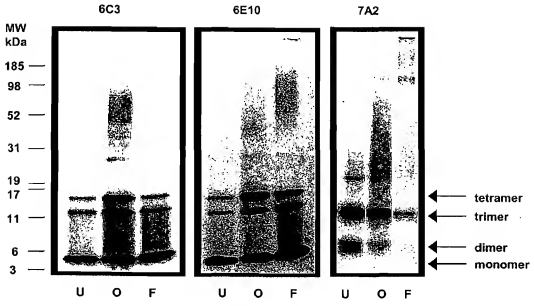

6C3
6E10
7A2
MW
kDa
185
98
52
31
19
17
11
6
3
U O F
U O F
U O F
tetramer
trimer
dimer
monomer

FIGURE 4

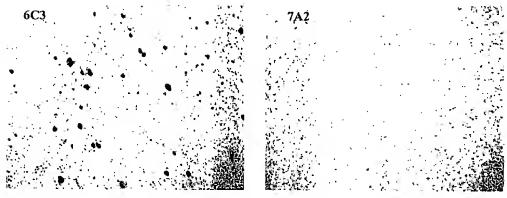


FIGURE 5

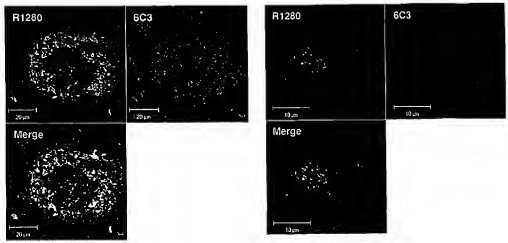
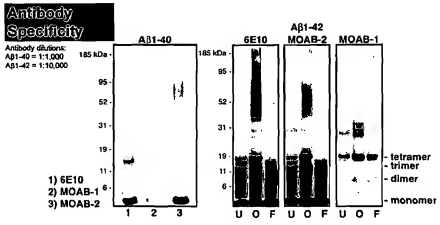


FIGURE 6


Antibody Specificity
Antibody dilutions:
Aβ1-40 = 1:1,000
Aβ1-42 = 1:10,000
Aβ1-40
Aβ1-42
6E10
MOAB-2
MOAB-1
185 kDa
95
52
31
19
11
6
1) 6E10
2) MOAB-1
3) MOAB-2
1 2 3
U O F U O F U O F
tetramer
trimer
dimer
monomer

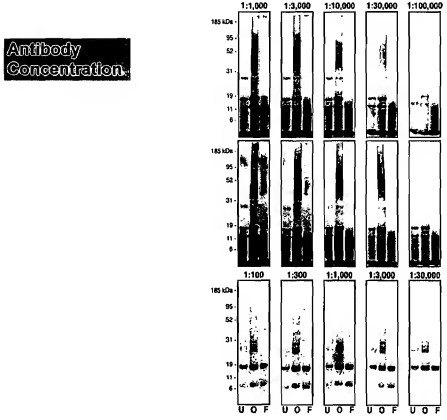
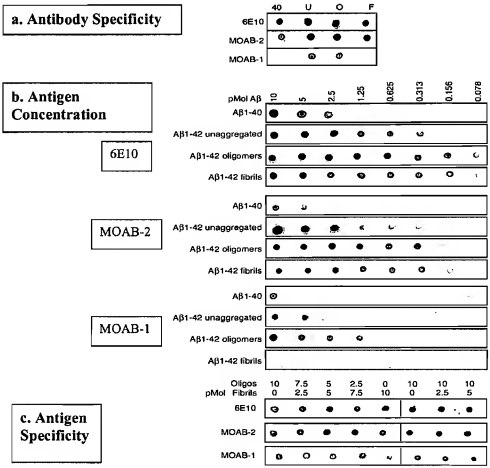

Antibody Concentration
1:1,000
1:3,000
1:10,000
1:30,000
1:100,000
1:100
1:300
1:1,000
1:3,000
1:30,000
185 kDa
95
52
31
19
11
6
U O F

FIGURE 7


a. Antibody Specificity
40 U O F
6E10
MOAB-2
MOAB-1
b. Antigen Concentration
pMol Aβ 10 5 2.5 1.25 0.625 0.313 0.156 0.078
6E10
MOAB-2
MOAB-1
Aβ1-40
Aβ1-42 unaggregated
Aβ1-42 oligomers
Aβ1-42 fibrils
c. Antigen Specificity
Oligos 10 7.5 5 2.5 0 10 10 10
pMol Fibrils 0 2.5 5 7.5 10 0 2.5 5
6E10
MOAB-2
MOAB-1

ANTIBODIES SPECIFIC FOR TOXIC AMYLOID BETA PROTEIN OLIGOMERS

[0001] The present invention claims priority to U.S. Pat. Appln. Ser. No. 60/491,725, filed Aug. 1, 2003, the disclosure of which is herein incorporated by reference in its entirety.

[0002] This invention was funded, in part, under NIH Grant AG13854. The government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention provides compositions and methods for diagnosing Alzheimer's disease (AD) and other conditions. In particular, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies proteolytically derived from the transmembrane amyloid precursor protein (APP) while not reacting with fibrillar amyloid β protein assemblies, monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies, and methods of use of thesc compositions in the diagnosis and treatment of Alzheimer's disease, as well as methods to monitor treatment and/or disease progression of AD in patients.

BACKGROUND OF THE INVENTION

[0004] AD is the fourth most common cause of death in the United States, next to heart disease, cancer and stroke. It presently afflicts more than four million people, and this number is expected to double during the next forty years with the aging of the population. AD is also the most common cause of chronic dementia, with approximately two million people in the United States suffering from dementia. At present, it is estimated that ten percent of the population older than 65 years of age have mild to severe dementia. This high prevalence, combined with the rate of growth of the elderly segment of the population, make dementia and particularly AD, important current public health problems.

[0005] To date, AD is the third most expensive disease in the United States, and costs approximately $100 billion each year. Costs associated with AD include direct medical costs such as nursing home care, direct non-medical costs such as in-home day care, as well as indirect costs such as lost patient and care-giver productivity. Medical treatment may have economic benefits by slowing the rate of cognitive decline, delaying institutionalization, reducing care-giver hours, and improving quality of life.

[0006] AD is a complex multi-genic neurodegenerative disorder characterized by progressive impairments in memory, behavior, language, and visuo-spatial skills, ending ultimately in death. Hallmark pathologies of AD include granulovascular neuronal degeneration, extracellular neuritic plaques with amyloid β protein deposits, intracellular neurofibrillary tangles and neurofibrillary degeneration, synaptic loss, and extensive neuronal cell death. It is now known that these histopathologic lesions of AD correlate with the dementia observed in many elderly people.

[0007] Research on the causes of and treatments for AD has led investigators down numerous avenues. Although many models have been proposed, no single model of AD satisfactorily accounts for all neuropathologic findings; nor do these models of AD satisfactorily account for the requirement of aging for disease onset. Cellular changes, leading to neuronal loss and the underlying etiology of the disease, remain unknown. Proposed causes include environmental factors (Perl, Environmental Health Perspective 63:149 [1985]), metal toxicity (Perl et al., Science 208:297 [1980]), defects in beta-amyloid protein metabolism (Shijo et al., Science 258:126 [1992]; and Kosik, Science 256:780 [1992]), and abnormal calcium homeostasis and/or calcium activated kinases (Mattson et al., J. Neuroscience 12:376 [1992]). The mechanisms of disease progression are equally unclear. Considerable human genetic evidence has implicated alterations in production or processing of the human amyloid precursor protein (APP) in the etiology of the disease. However, intensive research has proven that AD is a multifactorial disease with many different, perhaps overlapping, etiologies.

[0008] Early detection and identification of AD facilitate prompt, appropriate treatment and care. However, there is currently no laboratory diagnostic test for AD. Although studies have suggested that calcium imaging measurement in fibroblasts were of potential clinical use in diagnosing AD (Peterson et al., Neurobiology of Aging 9:261 [1988]; and Peterson et al., Proc. Natl. Acad. Sci. USA 83:7999 [1986]), other studies using similar cell lines and techniques have shown no difference in calcium levels in Alzheimer's and normal control fibroblasts (Borden et al., Neurobiology of Aging 13:33 [1991]). Thus, there remains a need for diagnostic methods for AD. In particular, reliable and cost-effective methods and compositions are needed to allow reliable diagnosis of AD.

SUMMARY OF THE INVENTION

[0009] The present invention provides compositions and methods for diagnosing Alzheimer's disease and other conditions. In particular, the present invention finds use with any condition (e.g., including but not limited to neurological conditions) that are directly or indirectly linked to the presence or absence of amyloid β protein assemblies. In particular, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies proteolytically derived from the transmembrane amyloid precursor protein (APP) while not reacting with fibrillar amyloid β protein assemblies, monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies, methods of use of these compositions in the diagnosis and treatment of Alzheimer's disease and other conditions, and methods to monitor treatment and/or disease progression of AD in patients, including methods of screening compounds for diagnostic and therapeutic application. For example, the present invention provides antibodies that may be used for therapeutic use through their ability to specifically bind to particular amyloid β protein assemblies. Following binding, the antibody bound complexes may be targeted with therapeutic compounds that are targeted to the complex or can be degraded and/or cleared by endogenous or exogenous routes. Compounds that find use in treating diseases and conditions can be screened for their ability to target, clear, or otherwise interact with amyloid β protein assemblies (e.g., by recognizing or competing with antibody binding). Thus, the present invention provides diagnostic, therapeutic, and

drug screening methods related to biological processes that are linked to the presence or absence of specific amyloid β protein assemblies.

[0010] In some embodiments, the present invention provides a composition comprising a purified monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins (although the present invention is not limited to any particular size). In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the amyloid β protein is the β1-42 protein. In still further embodiments, the amyloid β protein assemblies are neurotoxic.

[0011] The present invention also provides a hybridoma that secretes a monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the hybridoma secretes a monoclonal antibody that identifies oligomeric amyloid β proteins comprising the β1'-42 protein.

[0012] The present invention also provides methods for obtaining and isolating a hybridoma secreting a monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies, comprising the steps of: providing spleen cells immunized with an antigen comprising soluble, non-fibrillar oligomeric amyloid β protein assemblies; fusing the immunized cells with myeloma cells under hybridoma-forming conditions; and selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies comprising amyloid β proteins without recognizing fibrillar amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins.

[0013] The present invention further provides a method for producing a monoclonal antibody from a hybridoma secreting a monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies, comprising the steps of: culturing the hybridoma in an appropriate medium culture and recovering the monoclonal antibody excreted by the hybridoma, or, alternatively, implanting the hybridoma into the peritoneum of a mouse, and, when ascites have been produced by the animal, recovering the monoclonal antibody then formed from the ascites. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In still further embodiments, the present invention provides a monoclonal antibody produced by this method.

[0014] In some embodiments, the present invention provides a composition comprising a purified monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the amyloid β protein is the β1-42 protein.

[0015] The present invention also provides a hybridoma that secretes a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the hybridoma secretes a monoclonal antibody that identifies fibrillar amyloid β proteins comprising the β1-42 protein.

[0016] The present invention also provides methods for obtaining and isolating a hybridoma secreting a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies, comprising the steps of: providing spleen cells immunized with an antigen comprising fibrillar amyloid β protein assemblies; fusing the immunized cells with myeloma cells under hybridoma-forming conditions; and selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies comprising fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins.

[0017] The present invention further provides a method for producing a monoclonal antibody from a hybridoma secreting a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies, comprising the steps of: culturing the hybridoma in an appropriate medium culture and recovering the monoclonal antibody excreted by the hybridoma, or, alternatively, implanting the hybridoma into the peritoneum of a mouse, and, when ascites have been produced by the animal, recovering the monoclonal antibody then formed from the ascites. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In still further embodiments, the present invention provides a monoclonal antibody produced by this method.

[0018] The present invention further provides methods for detecting at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, comprising the steps of: providing a sample suspected of containing at least one soluble, non-fibrillar oligomeric amyloid β protein assembly and a monoclonal antibody that identifies soluble, non-fibrillar

oligomeric amyloid β protein assemblies, while not reacting with fibrillar amyloid β protein assemblies; contacting the sample with the antibody under conditions such that the antibody binds to the soluble, non-fibrillar oligomeric amyloid β protein assembly, to form an antigen-antibody complex; and detecting the presence of the antigen-antibody complex. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid. In some preferred embodiments, the sample is from a subject. In particularly preferred embodiments, the subject is a human. In further embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In some particularly preferred embodiments, the methods further comprise the step of diagnosing Alzheimer's disease. In additional particularly preferred embodiments, the Alzheimer's disease diagnosed using the methods of the present invention is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease. In some preferred embodiments, the methods further comprise the step of monitoring the efficacy of treatment of Alzheimer's disease.

[0019] In some preferred embodiments, the methods comprises an enzyme-linked immunosorbent assay. In particularly preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays. In alternative preferred embodiments, the antibody used in the methods of the present invention further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In some particularly preferred embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system. In alternative preferred embodiments, the methods further comprise at least one capture antibody, while in still further embodiments, the methods further comprise at least one detection antibody wherein the detection antibody is directed against the antibody directed against the soluble, non-fibrillar oligomeric amyloid β protein assemblies. In still further embodiments, the detection antibody further comprises at least one conjugated enzyme selected from the group consisting of horseradish peroxidase, alkaline phosphatase, urease, glucoamylase and β-galactosidase. In still further preferred embodiments, the methods further comprise the step of quantitating the at least one soluble, non-fibrillar oligomeric amyloid β protein assembly in the sample.

[0020] The present invention also provides kits for the detection of at least one soluble, non-fibrillar oligomeric amyloid β protein assembly comprising at least one antibody directed against at least one soluble, non-fibrillar oligomeric amyloid β protein assembly. In some embodiments, the kit comprises an immobilized support. In some preferred embodiments, the kit comprises an enzyme-linked immunosorbent assay kit. In still further embodiments, the kit further comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, an antibody-enzyme conjugate directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly, at least one capture antibody, 96-well microtiter plates precoated with the at least one capture antibody, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme-linked immunosorbent assay enzyme reaction substrate solution, and at least one amplifier system. In some preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system. The kits of the present invention may also contain any other useful components, including other antibodies (e.g., for detection of multiple different proteins) or other diagnostic reagents, therapeutic agents, instructions, education materials, and the like.

[0021] The present invention also provides methods for detecting at least one antibody directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly, comprising: a) providing a sample suspected of containing at least one antibody directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly and a detection antibody; b) contacting the sample with the soluble, non-fibrillar oligomeric amyloid β protein assembly, under conditions such that the antibody directed against a soluble, non-fibrillar oligomeric amyloid β protein assembly specifically binds to the soluble, non-fibrillar oligomeric amyloid β protein assembly to form an antigen-antibody complex; c) contacting the antigen-antibody complex with the detection antibody, under conditions such that the detection antibody specifically binds to the complex; and d) detecting the specific binding of the detection antibody to the antigen-antibody complex. In some preferred embodiments, the sample is selected from the group of samples consisting of blood, serous fluid, plasma, serum, cerebrospinal fluid, hybridoma conditioned culture medium, ascites fluid, and polyclonal antiserum. In some particularly preferred embodiments, the sample is from a subject, while in other preferred embodiments, the subject is human. In alternative preferred embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In still further preferred embodiments, the methods further comprise diagnosing Alzheimer's disease in the subject. In some preferred embodiments, the Alzheimer's disease is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease, and sporadic Alzheimer's disease. In preferred embodiments, the method comprises an enzyme-linked immunosorbent assay. In some preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent

assays. In still further embodiments, the detection antibody further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In additional embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system.

[0022] The present invention also provides kits for the detection of at least one antibody directed against at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, comprising at least one soluble, non-fibrillar oligomeric amyloid β protein assembly and at least one detection antibody. In some embodiments, the kit comprises an immobilized support. In some preferred embodiments, the kit is an enzyme-linked immunosorbent assay kit. In some preferred embodiments, the kit comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, at least one purified antibody directed against at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, at least one 96-well microtiter plate precoated with at least one soluble, non-fibrillar oligomeric amyloid β protein assembly, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme reaction substrate solution, and at least one amplifier system. In some particularly preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

[0023] The present invention further provides methods for detecting at least one fibrillar amyloid β protein assembly, comprising the steps of: providing a sample suspected of containing at least one fibrillar amyloid β protein assembly and a monoclonal antibody that identifies fibrillar amyloid β protein assemblies, while not reacting with soluble, non-fibrillar oligomeric amyloid β protein assemblies; contacting the sample with the antibody under conditions such that the antibody binds to the fibrillar amyloid β protein assembly to form an antigen-antibody complex; and detecting the presence of the antigen-antibody complex. In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. In some embodiments, the sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid. In some preferred embodiments, the sample is from a subject. In particularly preferred embodiments, the subject is a human. In further embodiments, the subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease. In some particularly preferred embodiments, the methods further comprise the step of diagnosing Alzheimer's disease. In additional particularly preferred embodiments, the Alzheimer's disease diagnosed using the methods of the present invention is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease. In some preferred embodiments, the methods further comprise the step of monitoring the efficacy of treatment of Alzheimer's disease.

[0024] In some preferred embodiments, the methods comprises an enzyme-linked immunosorbent assay. In particularly preferred embodiments, the enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays. In alternative preferred embodiments, the antibody used in the methods of the present invention further comprises a conjugated enzyme, wherein the conjugated enzyme is selected from the group of enzymes consisting of horseradish peroxidases, alkaline phosphatases, ureases, glucoamylases, and β-galactosidases. In some particularly preferred embodiments, the enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system. In alternative preferred embodiments, the methods further comprise at least one capture antibody, while in still further embodiments, the methods further comprise at least one detection antibody wherein the detection antibody is directed against the antibody directed against the fibrillar amyloid β protein assemblies. In still further embodiments, the detection antibody further comprises at least one conjugated enzyme selected from the group consisting of horseradish peroxidase, alkaline phosphatase, urease, glucoamylase and β-galactosidase. In still further preferred embodiments, the methods further comprise the step of quantitating the at least fibrillar amyloid β protein assembly in the sample.

[0025] The present invention also provides kits for the detection of at least one fibrillar amyloid β protein assembly comprising at least one antibody directed against at least one fibrillar amyloid β protein assembly. In some embodiments, the kit comprises an immobilized support. In some preferred embodiments, the kit comprises an enzyme-linked immunosorbent assay kit. In still further embodiments, the kit further comprises components selected from the group consisting of needles, sample collection tubes, 96-well microtiter plates, instructions, an antibody-enzyme conjugate directed against a fibrillar amyloid β protein assembly, at least one capture antibody, 96-well microtiter plates precoated with the at least one capture antibody, at least one coating buffer, at least one blocking buffer, distilled water, at least one enzyme-linked immunosorbent assay enzyme reaction substrate solution, and at least one amplifier system. In some preferred embodiments, the amplifier system is an alkaline phosphatase enzyme-linked immunosorbent assay amplifier system.

DESCRIPTION OF THE DRAWINGS

[0026] **FIG. 1** depicts hybridoma screening by antigen/antibody blotting.

[0027] **FIG. 2** shows an ELISA assay utilizing the monoclonal antibodies 6C3 and 7A2 (labeled "MOAB-2" and MOAB-1" respectively).

[0028] **FIG. 3** shows Western blot analysis of unaggregated, oligomeric, and fibrillar preparations of amyloid β proteins using the monoclonal antibodies 6C3, 6E10, and 7A2.

[0029] **FIG. 4** shows DAB staining and 10x light microscopy with monoclonal antibodies 6C3 and 7A2 in an AD brain.

[0030] **FIG. 5** shows laser scanning confocal microscopy of AD brain slices using monoclonal antibody 6C3 and polyclonal antibody R1280.

[0031] **FIG. 6** shows additional Western blot analysis of unaggregated, oligomeric, and fibrillar preparations of amyloid β proteins using the monoclonal antibodies 6C3, 6E10 and 7A2. Note: "MOAB-1" and "MOAB-2" correspond to 7A2 and 6C3 antibodies, respectively.

[0032] **FIG. 7** shows dot blot analysis of unaggregated, oligomeric, and fibrillar preparations of amyloid β proteins using the monoclonal antibodies 6C3, 6E10 and 7A2. Note: "MOAB-1" and "MOAB-2" correspond to 7A2 and 6C3 antibodies, respectively.

DEFINITIONS

[0033] To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

[0034] As used herein, the terms "peptide,""polypeptide" and "protein" all refer to a primary sequence of amino acids that are joined by covalent "peptide linkages." In general, a peptide consists of a few amino acids, typically from 2-50 amino acids, and is shorter than a protein. The term "polypeptide" encompasses peptides and proteins. In some embodiments, the peptide, polypeptide or protein is synthetic, while in other embodiments, the peptide, polypeptide or protein are recombinant or naturally occurring. A synthetic peptide is a peptide that is produced by artificial means in vitro (i.e., was not produced in vivo).

[0035] The terms "sample" and "specimen" are used in their broadest sense and encompass samples or specimens obtained from any source. As used herein, the term "sample" is used to refer to biological samples obtained from animals (including humans), and encompasses fluids, solids, tissues, and gases. In preferred embodiments of this invention, biological samples include cerebrospinal fluid (CSF), serous fluid, urine, saliva, blood, and blood products such as plasma, serum and the like. However, these examples are not to be construed as limiting the types of samples that find use with the present invention.

[0036] As used herein, the terms "soluble, non-fibrillar oligomeric amyloid β protein assembly,""oligomeric amyloid β protein assembly" and "oligomeric assembly" all refer to a protein assembly comprised of amyloid β proteins or peptides proteolytically derived from the transmembrane amyloid precursor protein (APP).

[0037] As used herein, the terms "fibrillar amyloid β protein assembly" and "fibrillar assembly" refers to a protein assembly comprised of amyloid β proteins or peptides proteolytically derived from the transmembrane amyloid precursor protein (APP).

[0038] As used herein, the term "oxidative stress" refers to the cytotoxic effects of oxygen radicals (i.e., superoxide anion, hydroxy radical, and hydrogen peroxide), generated as byproducts of metabolic processes that utilize molecular oxygen (See e.g., Coyle et al., Science 262:689-695 [1993]).

[0039] As used herein, the terms "host,""subject" and "patient" refer to any animal, including but not limited to, human and non-human animals (e.g. rodents, arthropods, insects [e.g., Diptera], fish [e.g., zebrafish], non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.), that is studied, analyzed, tested, diagnosed or treated. As used herein, the terms "host,""subject" and "patient" are used interchangeably.

[0040] As used herein, the terms "Alzheimer's disease" and "AD" refer to a neurodegenerative disorder and encompasses familial Alzheimer's disease and sporadic Alzheimer's disease. The term "familial Alzheimer's disease" refers to Alzheimer's disease associated with genetic factors (i.e., demonstrates inheritance) while "sporadic Alzheimer's disease" refers to Alzheimer's disease that is not associated with prior family history of the disease. Symptoms indicative of Alzheimer's disease in human subjects typically include, but are not limited to, mild to severe dementia, progressive impairment of memory (ranging from mild forgetfulness to disorientation and severe memory loss), poor visuo-spatial skills, personality changes, poor impulse control, poor judgement, distrust of others, increased stubbornness, restlessness, poor planning ability, poor decision making, and social withdrawal. In severe cases, patients lose the ability to use language and communicate, and require assistance in personal hygiene, eating and dressing, and are eventually bedridden. Hallmark pathologies within brain tissue include extracellular neuritic β-amyloid plaques, neurofibrillary tangles, neurofibrillary degeneration, granulovascular neuronal degeneration, synaptic loss, and extensive neuronal cell death.

[0041] As used herein, the term "early-onset Alzheimer's disease" refers to the classification used in Alzheimer's disease cases diagnosed as occurring before the age of 65. As used herein, the term "late-onset Alzheimer's disease" refers to the classification used in Alzheimer's disease cases diagnosed as occurring after the age of 65.

[0042] As used herein, the terms "subject having Alzheimer's disease" or "subject displaying symptoms or pathology indicative of Alzheimer's disease""subjects suspected of displaying symptoms or pathology indicative of Alzheimer's disease" refer to a subject that is identified as having or likely to have Alzheimer's disease based on known Alzheimer's symptoms and pathology.

[0043] As used herein, the term "subject at risk of displaying pathology indicative of Alzheimer's disease" refers to a subject identified as being at risk for developing Alzheimer's disease (e.g., because of a familial inheritance pattern of Alzheimer's disease in the subject's family).

[0044] As used herein, the term "lesion" refers to a wound or injury, or to a pathologic change in a tissue. For example, the amyloid plaque lesions observed in the brains of patients having Alzheimer's disease are considered the hallmark pathology characteristic of the disease.

[0045] As used herein, the term "antibody" (or "antibodies") refers to any immunoglobulin that binds specifically to an antigenic determinant, and specifically, binds to proteins identical or structurally related to the antigenic determinant that stimulated their production. Thus, antibodies are useful in assays to detect the antigen that stimulated their production. Monoclonal antibodies are derived from a single clone of B lymphocytes (i.e., B cells), and are generally homogeneous in structure and antigen specificity. Polyclonal antibodies originate from many different clones of antibody-producing cells, and thus are heterogenous in their structure

and epitope specificity, but all recognize the same antigen. In some embodiments, monoclonal and polyclonal antibodies are used as crude preparations, while in preferred embodiments, these antibodies are purified. For example, in some embodiments, polyclonal antibodies contained in crude antiserum are used. Also, it is intended that the term "antibody" encompass any immunoglobulin (e.g., IgG, IgM, IgA, IgE, IgD, etc.) obtained from any source (e.g., humans, rodents, non-human primates, lagomorphs, caprines, bovines, equines, ovines, etc.).

[0046] As used herein, the terms "auto-antibody" or "auto-antibodies" refer to any immunoglobulin that binds specifically to an antigen that is native to the host organism that produced the antibody (i.e., the antigen is directed against "self" antigens). The presence of auto-antibodies is referred to herein as "autoimmunity."

[0047] As used herein, the term "antigen" is used in reference to any substance that is capable of being recognized by an antibody. It is intended that this term encompass any antigen and "immunogen" (i.e., a substance that induces the formation of antibodies). Thus, in an immunogenic reaction, antibodies are produced in response to the presence of an antigen or portion of an antigen. The terms "antigen" and "immunogen" are used to refer to an individual macromolecule or to a homogeneous or heterogeneous population of antigenic macromolecules. It is intended that the terms antigen and immunogen encompass protein molecules or portions of protein molecules, that contains one or more epitopes. In many cases, antigens are also immunogens, thus the term "antigen" is often used interchangeably with the term "immunogen." In some preferred embodiments, immunogenic substances are used as antigens in assays to detect the presence of appropriate antibodies in the serum of an immunized animal.

[0048] As used herein, the terms "antigen fragment" and "portion of an antigen" and the like are used in reference to a portion of an antigen. Antigen fragments or portions typically range in size, from a small percentage of the entire antigen to a large percentage, but not 100%, of the antigen. However, in situations where "at least a portion" of an antigen is specified, it is contemplated that the entire antigen is also present (i.e., it is not intended that the sample tested contain only a portion of an antigen). In some embodiments, antigen fragments and/or portions thereof, comprise an "epitope" recognized by an antibody, while in other embodiments these fragments and/or portions do not comprise an epitope recognized by an antibody. In addition, in some embodiments, antigen fragments and/or portions are not immunogenic, while in preferred embodiments, the antigen fragments and/or portions are immunogenic.

[0049] The terms "antigenic determinant" and "epitope" as used herein refer to that portion of an antigen that makes contact with a particular antibody variable region. When a protein or fragment (or portion) of a protein is used to immunize a host animal, numerous regions of the protein are likely to induce the production of antibodies that bind specifically to a given region or three-dimensional structure on the protein (these regions and/or structures are referred to as "antigenic determinants"). In some settings, antigenic determinants compete with the intact antigen (i.e., the "immunogen" used to elicit the immune response) for binding to an antibody.

[0050] The terms "specific binding" and "specifically binding" when used in reference to the interaction between an antibody and an antigen describe an interaction that is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) on the antigen. In other words, the antibody recognizes and binds to a protein structure unique to the antigen, rather than binding to all proteins in general (i.e., non-specific binding).

[0051] As used herein the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

[0052] As used herein, the term "adjuvant" is defined as a substance that enhances the immunogenicity of a coadministered antigen. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant—or that the same adjuvant, once used, be used for all subsequent immunizations. The present invention contemplates many adjuvants, including but not limited to, keyhole limpet hemocyanin (KLH), agar beads, aluminum hydroxide or phosphate (alum), Freund's adjuvant (incomplete or complete), Quil A adjuvant and Gerbu adjuvant (Accurate Chemical and Scientific Corporation), and bacterins (i.e., killed preparations of bacterial cells, especially mycoplasma).

[0053] As used herein, the terms "purified" and "to purify" and "purification" refers to the removal or reduction of at least one contaminant from a sample. For example, antibodies are purified by removal of contaminating non-immunoglobulin proteins. Antibodies are also purified by the removal of immunoglobulin that does not bind to the target molecule. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule results in an increase in the percent of target-reactive immunoglobulins in the sample (i.e., "enrichment" of an antibody).

[0054] As used herein, the term "immunoassay" refers to any assay that uses at least one specific antibody for the detection or quantitation of an antigen. Immunoassays include, but are not limited to, Western blots, ELISAs, radio-immunoassays, and immunofluorescence assays. Furthermore, many different ELISA formats are known to those in the art, any of which will find use in the present invention. However, it is not intended that the present invention be limited to these assays. In additional embodiments, other antigen-antibody reactions are used in the present invention, including but not limited to "flocculation" (i.e., a colloidal suspension produced upon the formation of antigen-antibody complexes), "agglutination" (i.e., clumping of cells or other substances upon exposure to antibody), "particle agglutination" (i.e., clumping of particles coated with antigen in the presence of antibody or the clumping of particles coated with antibody in the presence of antigen), "complement fixation" (i.e., the use of complement in an antibody-antigen reaction method), and other methods commonly used in serology, immunology, immunocytochemistry, immunohistochemistry, and related fields.

[0055] The terms "Western blot,""Western immunoblot""immunoblot" and "Western" refer to the immunological analysis of protein(s), polypeptides or peptides that have been immobilized onto a membrane support. The proteins are first resolved by polyacrylamide gel electrophoresis (i.e.,

SDS-PAGE) to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to an antibody having reactivity towards an antigen of interest. The binding of the antibody (i.e., the primary antibody) is detected by use of a secondary antibody that specifically binds the primary antibody. The secondary antibody is typically conjugated to an enzyme that permits visualization of the antigen-antibody complex by the production of a colored reaction product or catalyzes a luminescent enzymatic reaction (e.g., the ECL reagent, Amersham).

[0056] As used herein, the term "ELISA" refers to enzyme-linked immunosorbent assay (or EIA). Numerous ELISA methods and applications are known in the art, and are described in many references (See, e.g., Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in Molecular Biomethods Handbook, Rapley et al. [eds.], pp. 595-617, Humana Press, Inc., Totowa, N.J. [1998]; Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]). In addition, there are numerous commercially available ELISA test systems.

[0057] One of the ELISA methods used in the present invention is a "direct ELISA," where an antigen (e.g., an oligomeric or a fibrillar amyloid β protein assembly) in a sample is detected. In one embodiment of the direct ELISA, a sample containing antigen is exposed to a solid (i.e., stationary or immobilized) support (e.g., a microtiter plate well). The antigen within the sample becomes immobilized to the stationary phase, and is detected directly using an enzyme-conjugated antibody specific for the antigen.

[0058] In an alternative embodiment, an antibody specific for an antigen is detected in a sample. In this embodiment, a sample containing an antibody (e.g., an anti-oligomeric or an anti-fibrillar assembly antibody) is immobilized to a solid support (e.g., a microtiter plate well). The antigen-specific antibody is subsequently detected using purified antigen and an enzyme-conjugated antibody specific for the antigen.

[0059] In an alternative embodiment, an "indirect ELISA" is used. In one embodiment, an antigen (or antibody) is immobilized to a solid support (e.g., a microtiter plate well) as in the direct ELISA, but is detected indirectly by first adding an antigen-specific antibody (or antigen), then followed by the addition of a detection antibody specific for the antibody that specifically binds the antigen, also known as "species-specific" antibodies (e.g., a goat anti-rabbit antibody), that are available from various manufacturers known to those in the art (e.g., Santa Cruz Biotechnology; Zymed; and Pharmingen/Transduction Laboratories).

[0060] In other embodiments, a "sandwich ELISA" is used, where the antigen is immobilized on a solid support (e.g., a microtiter plate) via an antibody (i.e., a capture antibody) that is immobilized on the solid support and is able to bind the antigen of interest. Following the affixing of a suitable capture antibody to the immobilized phase, a sample is then added to the microtiter plate well, followed by washing. If the antigen of interest is present in the sample, it is bound to the capture antibody present on the support. In some embodiments, a sandwich ELISA is a "direct sandwich" ELISA, where the captured antigen is detected directly by using an enzyme-conjugated antibody directed against the antigen. Alternatively, in other embodiments, a sandwich ELISA is an "indirect sandwich" ELISA, where the captured antigen is detected indirectly by using an antibody directed against the antigen, that is then detected by another enzyme-conjugated antibody that binds the antigen-specific antibody, thus forming an antibody-antigen-antibody-anti-body complex. Suitable reporter reagents are then added to detect the third antibody. Alternatively, in some embodiments, any number of additional antibodies are added as necessary, in order to detect the antigen-antibody complex. In some preferred embodiments, these additional antibodies are labelled or tagged, so as to permit their visualization and/or quantitation.

[0061] As used herein, the term "capture antibody" refers to an antibody that is used in a sandwich ELISA to bind (i.e., capture) an antigen in a sample prior to detection of the antigen. For example, in some embodiments, the monoclonal anti-oligomeric or anti-fibrillar assembly antibodies of the present invention serve as a capture antibody when immobilized in a microtiter plate well. This capture antibody binds oligomeric or fibrillar amyloid β protein assembly antigens present in a sample added to the well. In one embodimcnt of the present invention, biotinylated capture antibodies are used in the present invention in conjunction with avidin-coated solid support. Another antibody (i.e., the detection antibody) is then used to bind and detect the antigen-antibody complex, in effect forming a "sandwich" comprised of antibody-antigen-antibody (i.e., a sandwich ELISA).

[0062] As used herein, a "detection antibody" is an antibody that carries a means for visualization or quantitation, that is typically a conjugated enzyme moiety that typically yields a colored or fluorescent reaction product following the addition of a suitable substrate. Conjugated enzymes commonly used with detection antibodies in the ELISA include horseradish peroxidase, urease, alkaline phosphatase, glucoamylase and β-galactosidase. In some embodiments, the detection antibody is directed against the antigen of interest, while in other embodiments, the detection antibody is not directed against the antigen of interest. In some embodiments, the detection antibody is an antibody directed against an antibody directed against the antigen of interest. Alternatively, the detection antibody is prepared with a label such as biotin, a fluorescent marker, or a radioisotope, and is detected and/or quantitated using this label.

[0063] As used herein, the terms "reporter reagent,""reporter molecule,""detection substrate" and "detection reagent" are used in reference to reagents that permit the detection and/or quantitation of an antibody bound to an antigen. For example, in some embodiments, the reporter reagent is a calorimetric substrate for an enzyme that has been conjugated to an antibody. Addition of a suitable substrate to the antibody-enzyme conjugate results in the production of a colorimetric or fluorimetric signal (e.g., following the binding of the conjugated antibody to the antigen of interest). Other reporter reagents include, but are not limited to, radioactive compounds. This definition also encompasses the use of biotin and avidin-based compounds (e.g., including but not limited to neutravidin and streptavidin) as part of the detection system.

[0064] As used herein, the term "signal" is used generally in reference to any detectable process that indicates that a reaction has occurred, for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorimetric or colorimetric products/reagents will all find use with the present invention. In various embodiments of the present invention, the signal is assessed qualitatively, while in alternative embodiments, the signal is assessed quantitatively.

[0065] As used herein, the term "amplifier" is used in reference to a system that enhances the signal in a detection method, such as an ELISA (e.g., an alkaline phosphatase amplifier system used in an ELISA).

[0066] As used herein, the term "solid support" is used in reference to any solid or stationary material to which reagents such as antibodies, antigens, and other test components are attached. For example, in the ELISA method, the wells of microtiter plates provide solid supports. Other examples of solid supports include microscope slides, coverslips, beads, particles, cell culture flasks, gels, as well as many other suitable items.

[0067] As used herein, the term "kit" is used in reference to a combination of reagents and other materials that facilitate sample analysis. In some embodiments, the immunoassay kit of the present invention includes a suitable capture antibody, reporter antibody, antigen, detection reagents and amplifier system. Furthermore, in other embodiments, the kit includes, but is not limited to, components such as apparatus for sample collection, sample tubes, holders, trays, racks, dishes, plates, instructions to the kit user (including, for example, instructions and label as required by regulatory agencies), solutions or other chemical reagents, and samples to be used for standardization, normalization, and/or control samples.

[0068] As used herein, the term "in vitro" refers to an artificial environment and to processes or reactions that occur within an artificial environment. In vitro environments consist of, but are not limited to, controlled laboratory conditions. The term "in vivo" refers to the natural environment (e.g., an animal or a cell) and to processes or reactions that occur within that natural environment.

DETAILED DESCRIPTION OF THE INVENTION

[0069] The cellular processes that underlie the cognitive decline and amnestic dementia associated with AD remain poorly understood. Amyloid β protein is a peptide that is proteolytically derived from the transmembrane amyloid precursor protein (APP). Evidence from numerous studies supports the hypothesis that amyloid β protein accumulation is causally linked to AD. However a causal linkage between pathology, in terms of senile plaques composed primarily of deposited fibrillar amyloid β protein, and symptomology in terms of cognitive impairment and dementia, has not been forthcoming. An emerging hypothesis that reconciles this apparent disconnect focuses on small soluble assemblies of amyloid β protein.

[0070] Recent experimental evidence has demonstrated that these oligomeric conformations are directly involved in many of the destructive processes that result in neurodegeneration. Oligomeric amyloid β protein assemblies have been isolated from brain, plasma and CSF and soluble amyloid β protein concentrations in brain are correlated with the severity of AD. Furthermore, autosomal dominant mutations in the amyloid precursor protein (APP) and the presenilins (PS) increase the amount of amyloid β1-42, a proteolytic product of APP, and always result in AD. This provides powerful genetic evidence that some form of the amyloid β protein is involved in the disease process.

[0071] The role of amyloid β protein in the AD disease process has been put forth as the comprehensive "amyloid hypothesis" (Selkoe, J. Neuorpath. Exp. Neurol., 53: 438 [1991]). In it, it is stated that the production and deposition of amyloid β protein fibrils in plaques induces a neurotoxic event. Presumably, this initial event culminates in the intracellular accumulation of tau polymers as neurofibrillary tangles leading to neuronal dysfunction and death. The amyloid hypothesis gained wide acceptance when initial reports indicated that fibrillar amyloid β protein was cytotoxic in vitro (Pike et al., J. Neuroscience, 13: 1676 [1993]; Schenk et al., J. Med. Chem., 38: 4141 [1995]). However, the veracity of the amyloid hypothesis is challenged by a seeming disconnect between aspect of the plaque pathology and AD symptomatology.

[0072] In one of the first discoveries challenging this hypothesis, pathologists noted that the correlation between the number, location and distribution of senile plaques (amyloid load) and the degree of dementia as assessed neuropsychologically was poor at best (Swash et al., Connections, Cognition and Alzheimer's Disease, Springer-Verlag, Berlin/NY [1997]. Second, amyloid deposition in senile plaques is temporarily dissociated from cognitive defects in transgenic mouse models overexpressing APP and PS (Hsai et al., Proc. Natl. Acad. Sci., 96: 3228 [1999]; Holcomb et al., Nature Med., 4: 97 [1998]; Chui et al., Nature Med., 5: 560 [1999]; Moechars et al., J. Biol. Chem., 274: 6483 [1999]). Finally, several therapeutics designed to block fibril formation have been unsuccessful in delaying AD symptoms (Schenk et al., J. Med. Chem., 38: 4141 [1995]; Soto, Mol. Med. Today, 5: 343 [1999]).

[0073] On the other hand, several lines of evidence suggest that soluble oligomeric amyloid β protein species, as distinct from large, fibrillar aggregates, do correlate with AD pathology. The concentration of soluble amyloid β protein in the brain is highly correlated with disease severity (Lue et al., Amer. J. Path., 155: 853 [1999]; McLean et al., Ann Neurol., 46: 860 [1999]). Furthermore, results from in vitro experiments demonstrate that soluble oligomeric amyloid β proteins not only can readily form, but that these species are highly cytotoxic (Roher et al., J. Biol. Chem., 271: 20631 [1996]; Hartley et al., J. Neurosci., 19: 8876 [1999]; Lambert et al., Proc. Natl. Acad. Sci., 95: 6448 [1998]; Oda et al., Exp. Neurol., 136: 22 [1995]). For example, the process of amyloid β protein oligomerization is enhanced in the media of cells expressing the APP or PS mutations, providing a possible connection between toxic oligomer formation and AD genetics (Podlinsy et al., Biochemistry, 37: 3602 [1998]).

[0074] While a compelling argument can be made for the relevance of a toxic, diffusible amyloid β protein oligomer, the presence of this amyloid β protein species has not been demonstrated in normal or AD brain by immunohistochemistry. This is primarily due to a lack of antibodies that can

distinguish different conformational forms of the amyloid β protein. Hence, one of the primary limitations to properly dissecting the role of fibrillar versus oligomeric amyloid β protein assemblies has been the lack of conformational-specific antibodies that can distinguish between these two aggregates of the amyloid β protein.

[0075] Hence, although it is not clear whether amyloid β protein accumulation causes Alzheimer's disease or is an effect of Alzheimer's disease, considerable evidence has strengthened the view that amyloid β protein accumulation is the causative agent of Alzheimer's disease. However, it is not necessary to understand the cause or effect of amyloid β protein accumulation in Alzheimer's disease in order to practice the present invention, nor is it intended that the present invention be limited to any particular mechanism or mechanisms of disease genesis or toxicity. Indeed, an understanding of any of the mechanisms of pathogenesis are not necessary in order to use the present invention.

[0076] In some embodiments, the present invention provides monoclonal antibodies that specifically bind to soluble, non-fibrillar oligomeric amyloid β protein assemblies while not reacting with fibrillar amyloid β protein assemblies, and monoclonal antibodies that specifically bind to fibrillar amyloid β protein assemblies that do not react with soluble, non-fibrillar oligomeric amyloid β protein assemblies (e.g., as shown in Examples 2 and 3). In some embodiments, the soluble, non-fibrillar oligomeric amyloid β protein assemblies comprise 2-12 amyloid β proteins. In some embodiments, the fibrillar amyloid β protein assemblies comprise more than 12 amyloid β proteins. The present invention, however, is not limited by the number of amyloid β proteins present in the non-fibrillar oligomeric assemblies or fibrillar assemblies. In further embodiments, these antibodies are used to identify soluble, non-fibrillar oligomeric amyloid β protein assemblies or fibrillar amyloid β protein assemblies, respectively. However, it is not intended that the use of these antibodies be limited to identifying oligomeric and fibrillar forms of the amyloid β protein. For example, these antibodies may also be used to inhibit or to precipitate the assembly of amyloid β protein fibrils.

[0077] Additionally, the antibodies of the present invention find other uses, including enzyme-linked immunosorbent assays (ELISAs) (e.g., as shown in Example 3), Western blotting (e.g., as shown in Example 4), radioimmunoassays (RIAs), immunofluorescence assays (IFAs), immunoprecipitation, immunohistochemistry (e.g., as shown in Example 5), laser scanning confocal microscopy (e.g., as shown in Example 6) and clinical diagnostic applications using methods known in the art (See e.g., Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, Vol. 1-4, John Wiley & Sons, Inc., New York [1994]; and Laurino et al., Ann. Clin. Lab Sci., 29(3):158-166 [1999]).

[0078] It is not intended that the production of antibodies of the present invention be limited to any particular method. Indeed, it is contemplated that the antibodies be prepared by any suitable method. Numerous methods for the production and purification of monoclonal antibodies are well known in the art (See e.g., Sambrook et al. (eds.), Molecular Cloning, Cold Spring Harbor Laboratory Press [1989]; Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, p. 11.4.2-11.15.4, John Wiley & Sons, Inc., New York [1994]; Kohler and Milstein, Nature 256:495-497 [1975]; Kozbor et al., Immunol. Today 4:72 [1983]; and Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 [1985]).

[0079] In addition, in other embodiments, any suitable amyloid β protein or fragment thereof, is used as an immunogen. (e.g., generation of immunogens is described in Example 1). In some embodiments, the immunogen is native, while in other embodiments, the immunogen is synthetic (i.e., recombinant or produced by in vitro chemical synthesis). Similarly, it is not intended that the present invention be limited to any particular amyloid β protein-derived immunogen, immunization method, immunization schedule, animal species, test protocol for determining antibody production or antibody purification method.

[0080] In some embodiments, the monoclonal antibody preparation of the present invention is purified from crude antiserum, hybridoma or cell culture supernatant, ascites fluid, or other starting material using any conventional method. Such purification methods include, but are not limited to, protein A affinity, protein G affinity, ammonium sulfate precipitation, ion exchange chromatography, gel filtration, and immunoaffinity chromatography (See, e.g., Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; and Ausubel et al. (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]).

[0081] Clonal selection of hybridomas is performed by incubating supernatants from each clone in two ELISA wells, one with amyloid β protein oligomers attached and the other with fibrils attached (e.g., described in Example 2). Clonal supernatants from oligomer-immunized mice that are positive on the oligomer-attached plate but negative on the fibril-attached plate are selected for further subcloning. This dual selection protocol is repeated for screening fusion of splenocytes obtained from fibril-immunized mice.

[0082] The specificity of antibodies produced from hybridomas during the development of the present invention can be further characterized by antibody/antigen spotting and Western blotting (e.g., as described in Examples 2 and 3, respectively, below).

[0083] Experiments conducted during the course of development of the present invention showed an antigen/antibody screen yielding one oligomer-specific antibody. The oligomer-specific antibody (7A2) showed little recognition of fibrils by antigen/antibody blotting (e.g., see Example 2 and **FIG. 1**) and ELISA (e.g., see Example 3 and **FIG. 2**). By Western analysis, 7A2 detected oligomeric (primarily dimer, tetramer and larger oligomers between approximately 27 and 44 kDa) amyloid β protein assemblies (e.g., see Example 7, **FIGS. 6 and 7**), whereas 6E10 and 6C3 antibodies detected multiple forms of amyloid β protein1-42 including monomer, trimer, tetramer and oligomers between approximately 27 and 80 kDa. 7A2 oligomer specificity was retained over a wide range of antibody and antigen concentrations, and in the presence of increasing concentrations of fibrillar amyloid β protein1-42 (e.g., see Example 7, **FIGS. 6 and 7**). In sections from AD brain, little immunoreactivity of 7A2 antibody with fibrillar amyloid protein was detected (e.g., see Example 5 and **FIG. 4**). 6C3 antibody was used in

bright field immunohistochemistry and laser scanning confocal microscopy (LSCM) to detect structures resembling diffuse amyloid plaques not detected by AD polyclonal antibodies in hippocampal sections from the brains of neuropsychologically well-characterized AD patients (e.g., see Example 6 and **FIG. 5**).

[0084] It is known that oligomeric amyloid β protein assemblies are present in human blood and cerebrospinal fluid (CSF) of living subjects. It is contemplated that oligomeric amyloid β protein assemblies are also present in the blood, serous fluid and/or CSF of living subjects. It is contemplated that the presence of oligomeric amyloid β protein assemblies, or the presence of oligomeric assemblies above a threshold level, in these fluids is diagnostic of Alzheimer's disease. Thus, the present invention provides methods and compositions for the diagnosis and prognosis of Alzheimer's disease. Indeed, the present invention provides compositions and methods to analyze disease severity, and the efficacy of Alzheimer's disease therapies. It is contemplated that subjects identified as having higher levels of oligomeric amyloid β protein assemblies (e.g., in blood, serous fluid or CSF) have more advanced Alzheimer's disease than subjects showing lower levels of oligomeric amyloid β protein assemblies. It is contemplated that by monitoring the levels of oligomeric amyloid β protein assemblies in blood, serous fluid and/or CSF of patients undergoing treatment for Alzheimer's disease, determinations regarding the effectiveness of treatment regimes are possible. For example, reduced levels of oligomeric amyloid β protein assemblies over time indicate that the treatment used to treat a subject with Alzheimer's disease is effective.

[0085] It is contemplated that the present invention will find use in testing subjects such as those who have been previously diagnosed with Alzheimer's disease, those who are suspected of having Alzheimer's disease, and those at risk of developing Alzheimer's disease. For example, patients diagnosed with dementia, in particular, those patients who were previously clinically normal, are suitable subjects. However, it is not intended that the present invention be limited to use with any particular subject or patient types. The methods of the present invention are also useful for detecting early onset Alzheimer's disease and late onset Alzheimer's disease, as well as for detecting sporadic Alzheimer's disease and familial Alzheimer's disease.

[0086] The present invention also provides compositions and methods for the detection and quantitation (i.e., measurement) of oligomeric and fibrillar amyloid β protein assemblies in the blood, serous fluid and CSF. Standard techniques known in the art are easily adapted to quantitate the levels of circulating oligomeric and fibrillar amyloid β protein assemblies in blood, serous fluid and/or CSF samples, including but not limited to, ELISA.

[0087] Factors contributing to the success of the ELISA methods of the present invention include their sensitivity, versatility, long reagent shelf-life, ease of preparation of reagents, non-radioactive reagents, and assay speed. Furthermore, in some embodiments, the assay is quantitative. In addition, reagents and equipment designed specifically for use in ELISA protocols are readily available from numerous manufacturers, including Pierce Chemical Company, Bio-Rad, Dynatech Industries, GibcoBRL/Life Technologies, Fisher Scientific, and Promega.

[0088] Many ELISA applications and formats have been described. Various sources provide discussion of ELISA chemistry, applications, and detailed protocols (See e.g., Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in Molecular Biomethods Handbook, Rapley et al. [eds.], pp. 595-617, Humana Press, Inc., Totowa, NJ. [1998]; Harlow and Lane (eds.), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press [1988]; Ausubel et al. (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]; and Laurino et al., Ann. Clin. Lab Sci., 29(3):158-166 [1999]).

[0089] In preferred embodiments of the present invention, ELISA methods for quantitation of antigen are provided. In some of these methods, the antigen (e.g., oligomeric amyloid β protein) is first immobilized on a solid support (e.g., in a microtiter plate well). Detection and quantitation of the immobilized antigen is accomplished by use of an antibody-enzyme conjugate capable of binding to the immobilized antigen and producing a quantifiable signal. In some embodiments, the amount of antigen present is directly proportional to the amount of enzyme reaction product produced after the addition of an appropriate enzyme substrate.

[0090] As indicated previously, enzymes commonly used in ELISAs include horseradish peroxidase (HRPO), urease, alkaline phosphatase, glucoamylase and β-galactosidase. Protocols for the preparation of suitable antibody-enzyme conjugates are well known in the art. The present invention provides methods for the preparation of an antibody-enzyme (i.e., HRPO enzyme) conjugate that specifically recognizes the antigen of interest (i.e., oligomeric or fibrillar amyloid β protein assemblies) for use in immunoassay (e.g. ELISA) methods for detection of Alzheimer's disease. It is not intended that the present invention be limited to the antibody-enzyme conjugation method provided herein, as those of skill in the art will recognize other methods for antibody-enzyme conjugation that find use with the present invention.

[0091] Conjugation of enzymes to antibodies involves the formation of a stable, covalent linkage between an enzyme (e.g., HRPO or alkaline phosphatase) and the antibody (e.g., the monoclonal anti-oligomeric amyloid β protein assembly antibody or the monoclonal anti-fibrillar amyloid β protein assembly antibody), where neither the antigen-binding site of the antibody nor the active site of the enzyme is functionally altered.

[0092] The conjugation of antibody and HRPO is dependent on the generation of aldehyde groups by periodate oxidation of the carbohydrate moieties on HRPO (Nakane and Kawaoi, J. Histochem. Cytochem., 22:1084-1091 [1988]). Combination of these active aldehydes with amino groups on the antibody forms Schiff bases that, upon reduction by sodium borohydride, become stable.

[0093] Protocols to make antibody-enzyme conjugates using urease or alkaline phosphatase enzymes are also known in the art (Healey et al., Clin. Chim. Acta 134:51-58 [1983]; Voller et al., Bull. W. H. O., 53:55-65 [1976]; and Jeanson et al., J. Immunol. Methods 111:261-270 [1988]). For urease conjugation, cross-linking of the urease enzyme (e.g., Urease Type VII, Sigma No. U0376) and antibody using m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS) is achieved through benzoylation of free amino groups on the antibody. This is followed by thiolation of the

maleimide moiety of MBS by the cysteine sulfhydryl groups of urease. To prepare an alkaline phosphatase-antibody conjugate, a one-step glutaraldehyde method is the simplest procedure (Voller et al., Bull. W. H. O., 53:55-65 [1976]). This antibody-alkaline phosphatase conjugation protocol uses an enzyme immunoassay grade of the alkaline phosphatase enzyme.

[0094] The end product of an ELISA is a signal typically observed as the development of color or fluorescence. Typically, this signal is read (i.e., quantitated) using a suitable spectrocolorimeter (i.e., a spectrophotometer) or spectrofluorometer. The amount of color or fluorescence is directly proportional to the amount of immobilized antigen. In some embodiments of the present invention, the amount of antigen in a sample (e.g., the amount of oligomeric or fibrillar amyloid β protein assemblies in a blood or CSF sample) is quantitated by comparing results obtained for the sample with a series of control wells containing known concentrations of the antigen (i.e., a standard concentration curve). A negative control is also included in the assay system.

[0095] It is contemplated that any suitable chromogenic or fluorogenic substrates will find use with the enzyme-conjugated antibodies of the present invention. In some embodiments of the present invention, the substrate p-nitrophenyl phosphate (NPP) in diethanolamine is the preferred substrate for use in calorimetric ELISA methods, and 4-methylumbelliferyl phosphate (MUP) is the preferred alkaline phosphatase substrate in fluorometric ELISA methods.

[0096] The present invention provides various ELISA protocols for the detection and/or quantitation of oligomeric or fibrillar amyloid β protein assemblies in a sample. In one embodiment, the present invention provides a "direct ELISA" for the detection of oligomeric or fibrillar amyloid β protein assemblies in a sample. In some embodiments, the antigen of interest in a samplc (i.e., the oligomeric or fibrillar amyloid β protein assembly) is bound (along with unrelated antigens) to the solid support (e.g., a microtiter plate well). The immobilized antigen is then directly detected by the antigen-specific enzyme-conjugated antibody, also provided by the present invention. Addition of an appropriate detection substrate results in color development or fluorescence that is proportional to the amount of antigen present in the well.

[0097] In another embodiment, the present invention provides an indirect ELISA for the detection of antigen in a sample. In this embodiment, antigen of interest in a sample is immobilized (along with unrelated antigens) to a solid support (e.g., a microtiter plate well) as in the direct ELISA, but is detected indirectly by first adding an antigen-specific antibody, then followed by the addition of a detection antibody specific for the antibody that specifically binds the antigen, also known as "species-specific" antibodies (e.g., a goat anti-rabbit antibody), which are available from various manufacturers known to one in the art (e.g., Santa Cruz Biotechnology; Zymed; and Pharmingen/Transduction Laboratories).

[0098] In some embodiments, the concentration of sample added to each well is titrated, so as to produce an antigen concentration curve. In other embodiments, the concentration of conjugated antibody is titrated. Indeed, such titrations are typically performed during the initial development of ELISA systems.

[0099] In another embodiment, the present invention provides "sandwich ELISA" methods, in which the antigen in a sample is immobilized on the solid support by a "capture antibody" that has been previously bound to the solid support. In general, the sandwich ELISA method is more sensitive than other configurations, and is capable of detecting 0.1-1.0 ng/ml protein antigen. As indicated above, the sandwich ELISA method involves pre-binding the "capture antibody" which recognizes the antigen of interest (i.e., the oligomeric or fibrillar amyloid β protein assemblies) to the solid support (e.g. wells of the microtiter plate). In some embodiments, a biotinylated capture antibody is used in conjunction with avidin-coated wells. Test samples and controls are then added to the wells containing the capture antibody. If antigen is present in the samples and/or controls, it is bound by the capture antibody.

[0100] In some embodiments, after a washing step, detection of antigen that has been immobilized by the capture antibody is detected directly (i.e., a direct sandwich ELISA). In other embodiments detection of antigen that has been immobilized by the capture antibody is detected indirectly (i.e., an indirect sandwich ELISA). In the direct sandwich ELISA, the captured antigen is detected using an antigen-specific enzyme-conjugated antibody. In the indirect sandwich ELISA, the captured antigen is detected by using an antibody directed against the antigen, which is then detected by another enzyme-conjugated antibody which binds the antigen-specific antibody, thus forming an antibody-antigen-antibody—antibody complex. In both the direct and indirect sandwich ELISAs, addition of a suitable detection substrate results in color development or fluorescence that is proportional to the amount of antigen that is present in the well.

[0101] In the sandwich ELISA, the capture antibody used is typically different from the second antibody (the "detection antibody"). The choice of the capture antibody is empirical, as some pairwise combinations of capture antibody and detection antibody are more or less effective than other combinations. The same monoclonal antibody must not be used as both the capture antibody and the conjugated detection antibody, since recognition of a single epitope by the capture antibody will preclude the enzyme-conjugated detection antibody from binding to the antigen. However, in some embodiments, two different monoclonal antibodies that recognize different epitopes are used in this assay.

[0102] Furthermore, it is not intended that the present invention be limited to the direct ELISA and sandwich ELISA protocols particularly described herein, as the art knows well numerous alternative ELISA protocols that also find use in the present invention (See, e.g., Crowther, "Enzyme-Linked Immunosorbent Assay (ELISA)," in Molecular Biomethods Handbook, Rapley et al. [eds.], pp. 595-617, Humana Press, Inc., Totowa, N.J. [1998]; and Ausubel et al (eds.), Current Protocols in Molecular Biology, Ch. 11, John Wiley & Sons, Inc., New York [1994]). Thus, any suitable ELISA method including, but not limited to, competitive ELISAs also find use with the present invention.

[0103] In another embodiment, the present invention provides methods for the detection and quantitation of oligomeric or fibrillar amyloid β protein assembly reactive antibodies. Briefly, in some embodiments, variations of indirect ELISAs are used. In preferred embodiments, antigens (i.e., oligomeric or fibrillar amyloid β protein assemblies) are first

used to coat the wells of a 96-well microtiter plate. The test sample is then added to the antigen-coated wells. If the test sample contains oligomeric or fibrillar amyloid β protein assembly reactive antibodies, these antibodies specifically bind to the purified antigen coating the well. The oligomeric or fibrillar amyloid β protein assembly reactive antibodies are then visualized by the addition of a second detection antibody, where the detection antibody is coupled to an enzyme and is species-specific or isotype-specific for anti-oligomeric or anti-fibrillar amyloid β protein assembly antibody. As with all ELISA methods, appropriate negative and positive controls are included in order to ensure the reliability of the assay results.

[0104] It is contemplated that patients with Alzheimer's disease produce oligomeric or fibrillar amyloid β protein assembly-reactive auto-antibodies, and an ELISA to detect oligomeric or fibrillar amyloid β protein assembly reactive antibodies in such samples will find use in the diagnosis of Alzheimer's disease. It is further contemplated that the presence of anti-oligomeric or anti-fibrillar amyloid β protein assembly auto-antibodies in a patient is diagnostic of Alzheimer's disease.

[0105] It is also contemplated that the present invention will find use in detection of oligomeric or fibrillar amyloid β protein assembly reactive antibodies in various other settings (e.g., in the screening of monoclonal hybridoma culture supernatants [i.e., conditioned hybridoma culture medium], ascites fluid and/or polyclonal antisera).

[0106] The present invention also provides ELISA amplification systems. These embodiments produce at least 10-fold, and more preferably, a 500-fold increase in sensitivity over traditional alkaline phosphatase-based ELISAs. In one preferred embodiment of the ELISA amplification protocol, bound alkaline phosphatase acts on an NADPH substrate, whose reaction product initiates a secondary enzymatic reaction resulting in a colored product. Each reaction product from the first reaction initiates many cycles of the second reaction in order to amplify the signal (See e.g., Bio-Rad ELISA Amplification System, Cat. No. 19589-019).

[0107] The present invention also provides ELISA kits for the detection of antibodies and/or antigen. In addition, in some embodiments, the kits are customized for various applications. However, it is not intended that the kits of the present invention be limited to any particular format or design. In some embodiments, the kits of the present invention include, but are not limited to, materials for sample collection (e.g., spinal and/or venipuncture needles), tubes (e.g., sample collection tubes and reagent tubes), holders, trays, racks, dishes, plates (e.g., 96-well microtiter plates), instructions to the kit user, solutions or other chemical reagents, and samples to be used for standardization, and/or normalization, as well as positive and negative controls. In particularly preferred embodiments, reagents included in ELISA kits specifically intended for the detection of oligomeric or fibrillar amyloid β protein assemblies or anti-oligomeric or anti-fibrillar amyloid β protein assembly antibodies include control oligomeric amyloid β protein assemblies, anti-oligomeric and/or anti-fibrillar amyloid β protein assembly antibodies, anti-oligomeric and/or anti-fibrillar amyloid β protein assembly antibody-enzyme conjugate, 96-well microtiter plates precoated with control RA-NDA peptide, suitable capture antibody, 96-well microtiter plates precoated with a suitable oligomeric and/or anti-fibrillar amyloid β protein assembly capture antibody, buffers (e.g., coating buffer, blocking buffer, and distilled water), enzyme reaction substrate and premixed enzyme substrate solutions.

[0108] It is contemplated that the compositions and methods of the present invention will find use in various settings, including research and clinical diagnostics. For example, the anti-oligomeric and/or anti-fibrillar amyloid β protein assembly antibodies of the present invention also find use in studies of APP metabolism and in in situ hybridization studies of brain tissue sections to observe Alzheimer's disease pathology. In addition, methods to quantitate oligomeric and/or fibrillar amyloid β protein assemblies in samples find use in monitoring and/or determining the effectiveness of Alzheimer's disease treatment, as it is contemplated that decreasing levels of oligomeric amyloid β protein assemblies in a subject's samples over time indicates the effectiveness of an Alzheimer's disease treatment. Uses of the compositions and methods provided by the present invention encompass human and non-human subjects and samples from those subjects, and also encompass research as well as diagnostic applications. Thus, it is not intended that the present invention be limited to any particular subject and/or application setting.

EXPERIMENTAL

[0109] The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

[0110] In the experimental disclosure which follows, the following abbreviations apply: degree. C. (degrees Centigrade); cm (centimeters); g (grams); 1 or L (liters); .mu.g (micrograms); .mu.l (microliters); .mu.m (micrometers); .mu.M (micromolar); lmol (micromoles); mg (milligrams); ml (milliliters); mm (millimeters); mM (millimolar); mmol (millimoles); M (molar); mol (moles); ng (nanograms); nm (nanometers); nmol (nanomoles); N (normal); pmol (picomoles); Aldrich (Sigma/Aldrich, Milwaukee, Wis.); Amersham (Amersham/Pharmacia Biotech, Piscataway, N.J.); Bio-Rad (Bio-Rad Laboratories, Hercules, Calif.), Boehringer Mannheim (Boehringer Mannheim Corporation, Indianapolis, Ind.); Dynex (Dynex Technologies, Inc., Chantilly, Va.); Fisher Scientific (Fisher Scientific, Pittsburgh, Pa.), GiboBRL/Life Technologies (GibcoBRL/Life Technologies, Gaithersburg, Md.), Oncogene Research Products (Oncogene Research Products, Cambridge, Mass.); Pharmingen/Transduction Laboratories (Pharmingen/Transduction Laboratories/Becton Dickinson Company, San Diego, Calif.); Pierce Chemical Company (Pierce Chemical Company, Rockford, Ill.); Promega (Promega Corporation, Madison, Wis.); Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.); Sigma (Sigma Chemical Co., St. Louis, Mo.); and Zymed (Zymed Laboratories, Inc., South San Francisco, Calif.).

Example 1

Materials and Methods

[0111] Generation of fibrillar or oligomeric immunogens for generation of hybridomas. Pretreated stocks of amyloid

β protein stored as HFIP films are monomerized in DMSO, then aggregated in dilute acid at low salt (10 mM HCL) to produce fibrillar amyloid β protein, or, in cell culture media (phenyl-free F12, Gibco BRL) containing physiologic salt and pH levels to produce oligomeric structures. The amyloid β protein fibrils produced under the acidic conditions have diameters that measure approximately ~4 nm in z-height and extend for several microns. Oligomers produced in cell culture media range in size from ~2 nm in z-height.

[0112] Immunizations. Female Balb/c mice are immunized with amyloid β protein oligomers or fibrils produced as described above. The immunogens employed are suspended in Freunds Incomplete Adjuvant at a concentration of 1 μg/μl. A total of 200 μg is injected subcutaneously every 2 weeks until the serum titer of the mouse is half-maximal at a dilution of 2×10^{-4} as judged by ELISA with 50 ng of amyloid β protein oligomers or fibrils attached per well in the solid phase.

[0113] Hybridoma production and clonal selection. Once the desired serum titer is attained, immune spleens are removed from the mice, dissociated, and fused with SP2/o myeloma cells. The resultant cell suspension is plated in 96 well plates, HAT selected and cultured for 10-14 days to allow clonal growth. Initial clonal selection is performed by incubating supernatants from each clone in two ELISA wells, one with amyloid β protein oligomers attached and the other with fibrils attached. Clonal supernatants from oligomer-immunized mice that are positive on the oligomer-attached plate but negative (or exhibit a two-fold or greater signal diminution) on the fibril-attached plate are selected for further subcloning. This dual selection protocol is repeated for screening fusion of splenocytes obtained from fibril-immunized mice. In this case, clones are selected that bind to the fibrils but not to the oligomers.

[0114] Subcloning and antibody production. Mother clones are subcloned 3-4 times to assure monoclonality and allow the hybrids to stabilize. Antibodies are isotyped and the stable clones are adapted to serum-free medium and placed in a bioreactor for antibody expression. Purified, homogenous monoclonal antibodies are then stored at 1 mg/ml in borate buffered saline containing 50% glycerol.

Example 2

Screening of Hybridoma Supernatants by Antigen/Antibody Blotting

[0115] In order to determine the specificity of the antibodies made by the hybridomas, supernatants of the hybridomas were screened by antigen/antibody blotting. 5 μM amyloid β protein1-42 oligomer or fibril solutions were incubated with Immobilon-P membranes at room temperature for 30 minutes. Following rinsing and blocking, hybridoma supernatant was spotted onto membrane with a 96-pin replicator.

[0116] This method identified several hybridomas making antibodies which appeared specific for oligomer amyloid β protein assemblies and not the fibrillar form (**FIG. 1**). Monoclonal antibodies from the hybridoma 7A2 are oligomer-specific and show little recognition of fibrils by antigen/antibody blotting. In contrast, antibodies from the hybridoma 6C3 do not demonstrate specificity for oligomers or fibrils by antigen/antibody blotting.

Example 3

ELISA Titer: Oligomer-Versus Fibril Specificity for 6C3 and 7A2

[0117] Antibodies from hybridomas 7A2 and 6C3 were purified to homogeneity and further characterized in an ELISA assay. Serial dilutions of the purified antibodies were incubated with 25 ng of fibrillar or oligomeric amyloid β protein assemblies in the solid phase. 7A2 antibodies do not recognize fibrils by antigen/antibody blotting (**FIG. 1**). Additionally, standard ELISA shows that 7A2 antibodies display significant affinity for oligomeric assemblies while not displaying a similar affinity for fibrillar assemblies (**FIG. 2**). In contrast, although 6C3 does not demonstrate specificity for oligomers or fibrils by antigen/antibody blotting, standard ELISA shows 6C3 antibodies display some preference for oligomeric assemblies over that of fibrillar assemblies (**FIG. 2**).

Example 4

Western Blot Analysis Using 7A2 and 6C3 Antibodies

[0118] To further characterize the 7A2 and 6C3 antibodies, Western blot analysis was performed. Unaggregated (U), oligomeric (O), and fibrillar (F) amyloid β protein1-42 were run on 12% NUPAGE Bis-Tris gels under non-reducing conditions. The blots were probed with purified 6C3 (1:10,000), 6E10 (1:5000), or 7A2 (1:1000) monoclonal antibodies.

[0119] The oligomer-specific antibody (7A2) shows little recognition of fibrils by antigen/antibody blotting (**FIG. 1**) and ELISA (**FIG. 2**). By Western analysis of SDS-PAGE, 7A2 detects primarily dimer and trimer but no amyloid β protein monomers in unaggregated or oligomeric samples, and little immunoreactivity is detected in the fibril samples (**FIG. 3**). In contrast, 6C3 demonstrates a slight selectivity for oligomers over fibrils by ELISA (**FIG. 2**), however no differences are detected in Western blots between unaggregated, oligomer or fibril samples; here it reacts much like other amyloid β protein monoclonal antibodies such as 6E10 and 4G8 (**FIG. 3**).

Example 5

Bright Field Immunohistochemistry

[0120] The monoclonal antibodies 7A2 and 6C3 were analyzed on sections of human brain using standard peroxidase-based immunohistochemistry (**FIG. 4**). Tissue sections from the superior parietal lobule were obtained from well-characterized AD patients. The sections were stained with purified 6C3 antibody at a 1:20,000 dilution and with the tissue culture supernatant of 7A2 at a 1:1000 dilution. The magnification is 10x. In sections from AD brain, little 7A2 immunoreactivity with fibrillar amyloid plaques is detected (**FIG. 4**).

Example 6

Laser Scanning Confocal Microscopy (LSCM) of AD Brain Slices

[0121] In order to determine whether an antibody specific for soluble oligomers coexists in the same cells, plaques or

areas in the neuropil as Thioflavin S-positive plaques, double or triple immunofluorescence assessment using LSCM is useful. Sections from the same brain regions as those studied using the peroxidase procedure above are used. In this procedure, the primary antibodies are each detected with a different fluorochrome (either directly conjugated to the primary antibody or to a species-specific or isoform-specific secondary antibody). The z-sections obtained from a series of confocal images can be stacked and both fluorescence channels combined following pseudo-coloring. These stacks can then be rotated to view the three-dimensional image from a number of angels. For double immunofluorescence confocal microscopy, the co-localization feature of the software Metamorph is used to establish the percent co-localization between two fluorochromes from representative digital images.

[0122] A problem frequently encountered in any immunofluorescence studies on aged human brains is the presence of significant amounts of autofluorescent lipofuscin that can be confused with the yellow fluorescence seen during colocalization. In order to minimize this problem, sections are placed in a potassium permanganate solution (0.25% in phosphate buffered saline) for 20 minutes, after which a brown color develops. The sections are washed in phosphate buffered saline for two minutes, and then treated with a solution of 1.0 g % potassium metabisulfite and 1.0 g % oxalic acid in phosphate buffered saline until the brown color dissipates, typically occurring in 1-6 minutes. Finally, the sections are washed three times in phosphate buffered saline for two minutes each. Alternatively, Cy 5, a fluorochrome that emits in the infrared range can be used, thereby circumventing the autofluorescence of lipofuscin that does not emit light in the infrared spectrum of light.

[0123] The monoclonal antibody 6C3 and an amyloid β protein polyclonal antibody (R1280) were used to immunohistochemically stain an amyloid plaque and diffuse amyloid β protein deposits in the temporal lobe from an AD patient. 6C3 detected diffuse amyloid plaque-like structures not detected by AD polyclonal antibodies (**FIG. 5**).

Example 7

Western Blot and Dot Blot Analysis Using 7A2 and 6C3 Antibodies

[0124] To still further characterize the 7A2 and 6C3 antibodies, Western blot analysis was repeated on unaggregated (U), oligomeric (O), and fibrillar (F) amyloid β protein1-42 as in Example 4. 1100 pMol amyloid β protein1-40 or 200 pMol amyloid β protein1-42 were run on 4-12% BIS-TRIS NuPAGE gels, transferred to PVDF membrane and probed with each respective antibody. In this experiment, the 7A2 antibody only detected oligomeric amyloid β protein1-42 assemblies (primarily dimer, tetramer and larger oligomers between approximately 27 and 44 kDa), whereas 6E10 and 6C3 detected multiple forms of amyloid β protein1-42 including monomer, trimer, tetramer and oligomers between approximately 27 and 80 kDa (**FIG. 6**). The data presented in **FIG. 6** accurately reflects the identity of the bands. To further confirm the data, a range of antibody concentrations was hybridized to amyloid β protein1-42 transferred to PVDF. The oligomer specificity of 7A2 was retained over a wide range of antibody concentrations (**FIG. 6**). Note: "MOAB-1" and "MOAB-2" in **FIG. 6** correspond to 7A2 and 6C3 antibodies, respectively.

[0125] Further confirmation of the specificity of 7A2 for oligomeric amyloid β protein1-42 was achieved using dot blot analysis of different conformations of amyloid β protein immobilized on nitrocellulose. In **FIG. 7***a*, 10 pMol of amyloid β protein1-40, unaggregated amyloid β protein1-42, oligomeric amyloid β protein1-42, or fibrillar amyloid β protein1-42 were spotted on nitrocellulose and probed with 6E10, 6C3 or 7A2 antibodies. In **FIG. 7***b*, a dilution series of amyloid β protein antigens was probed with 6E10, 6C3 or 7A2. In **FIG. 7***c*, different ratios of oligomeric and fibrillar amyloid β protein1-42 were co-applied to nitrocellulose membrane and probed with 6E10, 6C3 or 7A2 antibodies. The dot blot analysis produced results consistent with both Western blot and ELISA results. 7A2 displayed greater affinity for oligomeric amyloid β protein1-42 as compared to amyloid β protein1-40, unaggregated or fibrillar amyloid β protein1-42. This selective high affinity interaction was maintained over a range of antigen concentrations. 6C3 and 6E10 were more immunoreactive to amyloid β protein1-42 than amyloid β protein1-40 but did not differentiate between oligomeric or fibrillar assemblies of amyloid β protein1-42. 7A2 also demonstrated specificity for oligomeric species in the presence of increasing concentrations of co-deposited fibrillar amyloid β protein1-42.

[0126] All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the present invention.

What is claimed is:

1. A composition comprising a purified monoclonal antibody that identifies soluble, non-fibrillar oligomeric amyloid β protein assemblies.

2. The composition of claim 1, wherein said amyloid β protein is the β1-42 protein.

3. A hybridoma that secretes said monoclonal antibody of claim 1.

4. A method for obtaining and isolating a hybridoma secreting said monoclonal antibody of claim 1, comprising:

a) providing spleen cells immunized with an antigen comprising soluble, non-fibrillar oligomeric amyloid β protein assemblies, wherein said antigen is recognized by said monoclonal antibody of claim 1;

b) fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and

c) selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies comprising amyloid β proteins without recognizing fibrillar amyloid β protein assemblies.

5. A composition comprising a purified monoclonal antibody suitable for identification of fibrillar amyloid β protein assemblies that does not identify soluble, non-fibrillar oligomeric amyloid β protein assemblies.

6. The composition of claim 5, wherein said amyloid β protein is the β1-42 protein.

7. A hybridoma that secretes said monoclonal antibody of claim 5.

8. A method for obtaining and isolating a hybridoma secreting said monoclonal antibody of claim 5, comprising:

a) providing spleen cells immunized with an antigen comprising fibrillar amyloid β protein assemblies, wherein said antigen is recognized by said monoclonal antibody of claim 5;

b) fusing said immunized cells with myeloma cells under hybridoma-forming conditions; and

c) selecting those hybridomas that secrete monoclonal antibodies that specifically recognize assemblies containing amyloid β proteins without recognizing soluble, non-fibrillar oligomeric amyloid β protein assemblies.

9. A method for detecting at least one amyloid β protein assembly, comprising the steps of:

a) providing

i) a sample from a subject suspected of containing at least one amyloid β protein assembly; and

ii) an antibody that identifies amyloid β protein assemblies;

b) contacting said sample with said antibody under conditions such that said antibody binds to said amyloid β protein assembly, forming an antigen-antibody complex; and

c) detecting the presence of said antigen-antibody complex.

10. The method of claim 9, wherein said at least one amyloid β protein assembly comprises soluble, non-fibrillar oligomeric amyloid β protein comprising 2-12 amyloid β proteins.

11. The method of claim 9, wherein said at least one amyloid β protein assembly comprises fibrillar amyloid β protein comprising more than 12 amyloid β proteins.

12. The method of claim 9, wherein said sample is selected from the group consisting of blood, plasma, serum, serous fluid, and cerebrospinal fluid.

13. The method of claim 9, wherein said subject is selected from the group consisting of subjects displaying pathology resulting from Alzheimer's disease, subjects suspected of displaying pathology resulting from Alzheimer's disease, and subjects at risk of displaying pathology resulting from Alzheimer's disease.

14. The method of claim 9, further comprising the step of diagnosing Alzheimer's disease, wherein said Alzheimer's disease is selected from the group consisting of late onset Alzheimer's disease, early onset Alzheimer's disease, familial Alzheimer's disease and sporadic Alzheimer's disease.

15. The method of claim 9, wherein said detecting at least one amyloid β protein assembly comprises an enzyme-linked immunosorbent assay, wherein said enzyme-linked immunosorbent assay is selected from the group consisting of direct enzyme-linked immunosorbent assays, indirect enzyme-linked immunosorbent assays, direct sandwich enzyme-linked immunosorbent assays, indirect sandwich enzyme-linked immunosorbent assays, and competitive enzyme-linked immunosorbent assays.

16. The method of claim 9, further comprising the step of quantitating said at least one amyloid β protein assembly in said sample.

17. The method of claim 15, wherein said enzyme-linked immunosorbent assay further comprises an alkaline phosphatase amplification system.

18. The method of claim 15, further providing at least one capture antibody.

19. The method of claim 18, further providing at least one detection antibody.

* * * * *

Exhibit 3

In connection with Application No. 09/402,820

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Conformation-sensitive Antibodies against Alzheimer Amyloid-β by Immunization with a Thioredoxin-constrained B-cell Epitope Peptide*

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Immunotherapy against the amyloid-β (Aβ) peptide is a valuable potential treatment for Alzheimer disease (AD). An ideal antigen should be soluble and nontoxic, avoid the C-terminally located T-cell epitope of Aβ, and yet be capable of eliciting antibodies that recognize Aβ fibrils and neurotoxic Aβ oligomers but not the physiological monomeric species of Aβ. We have described here the construction and immunological characterization of a recombinant antigen with these features obtained by tandem multimerization of the immunodominant B-cell epitope peptide Aβ1–15 (Aβ15) within the active site loop of bacterial thioredoxin (Trx). Chimeric Trx(Aβ15)$_n$ polypeptides bearing one, four, or eight copies of Aβ15 were constructed and injected into mice in combination with alum, an adjuvant approved for human use. All three polypeptides were found to be immunogenic, yet eliciting antibodies with distinct recognition specificities. The anti-Trx(Aβ15)$_4$ antibody, in particular, recognized Aβ42 fibrils and oligomers but not monomers and exhibited the same kind of conformational selectivity against transthyretin, an amyloidogenic protein unrelated in sequence to Aβ. We have also demonstrated that anti-Trx(Aβ15)$_4$, which binds to human AD plaques, markedly reduces Aβ pathology in transgenic AD mice. The data indicate that a conformational epitope shared by oligomers and fibrils can be mimicked by a thioredoxin-constrained Aβ fragment repeat and identify Trx(Aβ15)$_4$ as a promising new tool for AD immunotherapy.

Antipeptide antibodies are valuable tools for probing structure-function relationships in proteins as well as for therapeutic and diagnostic applications. When immunotherapy is the ultimate goal, the sequence span of the peptide, and thus the nature of the epitope(s) associated with it, as well as the conformational selectivity of the resulting antibodies may also be dictated by safety reasons. This is the case of the amyloid-β (Aβ)[2] peptide, a major neuropathological hallmark (1–3) and a promising immunotherapeutic target of Alzheimer disease (AD) (4–7). Following the encouraging results obtained with Aβ42 vaccination in transgenic mouse models of AD (8–10), a Phase II clinical trial utilizing preaggregated Aβ42 as an antigen and the QS-21 saponin as an immunoadjuvant (AN-1792 vaccine) was halted due to the occurrence of meningoencephalitis in ~6% of treated patients (11–13). T-cell-mediated autoimmunity produced by the C-terminal portion of Aβ (14) along with a predominantly pro-inflammatory (T helper 1) immunoresponse and cross-reactivity with the presumably physiological monomeric forms of Aβ (Aβ38–43 peptides plus the amyloid-β precursor protein have been hypothesized as the main causes of these adverse effects (15–17). Of further concern is the fact that not only plaques but also soluble Aβ oligomers should be bound by a therapeutically effective antibody (18–20). The latter represent an intermediate conformation prior to fibril formation and are presently considered as the most proximate causative agents of AD (21–24).

Some of the above limitations have been overcome through the development of alternative immunogens relying on N-terminally located B-cell epitope-bearing fragments rather than on full-length Aβ (25–33). Also important in this regard is the availability of Aβ peptide formulations capable of eliciting antibodies that recognize specific assembly states of Aβ42 (19, 34–36). For example, antibodies binding to oligomeric and fibrillar (but not to monomeric) Aβ, have been produced by using purified Aβ42 oligomers (19) or nitrated Aβ40 (35) as antigens, whereas antibodies selectively recognizing soluble Aβ oligomers have been obtained by immunization with Aβ40 immobilized onto gold nanoparticles (36). Interestingly, the latter antibodies bind not only to Aβ oligomers but also to the oligomeric forms of various Aβ-unrelated amyloidogenic polypeptides, suggesting that a common (as yet unidentified) conformation-dependent structure is shared by soluble oligomers regardless of their sequence (36).

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[2] The abbreviations used are: Aβ, amyloid-β; AD, Alzheimer disease; APP, amyloid-β precursor protein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Trx, bacterial thioredoxin; TTR, human transthyretin.

A similar conformational selectivity has not been achieved so far with a T-cell epitope lacking a fragment of Aβ. Despite the lack of detailed information on the molecular basis of the conformational mimics generated by chemical modification or surface immobilization of the Aβ peptide, it is clear that some kind of structural constraining is involved. We thus reasoned that a similar conformational effect, besides an enhanced peptide stabilization and immunogenicity (37, 38), might be obtained with the use of a scaffold protein, such as thioredoxin, with the ability to constrain the structure of short peptides inserted within its surface-exposed active site loop (38–40) and to stimulate T-cell proliferation (41, 42). This kind of recombinant antigen would be highly desirable because of its expected safety, ease of construction, and large scale production in a chemically homogeneous form.

We have shown here that antibodies recognizing Aβ42 oligomers and fibrils (but not monomers) are produced by immunization with a 4-fold repeat of the Aβ15 peptide bearing an interposed three-amino-acid linker and arranged in tandem within the display site of bacterial thioredoxin (Trx). As revealed by comparison with $Trx(Aβ15)_n$ antigens bearing one or eight copies of Aβ15, conformational selectivity critically depends on Aβ15 multiplicity and on the use of multipeptide insertions fitting the structural constraining capacity of Trx. We have further documented the ability of the anti-$Trx(Aβ15)_4$ antibody to bind Aβ aggregates in human brain and to ameliorate AD pathology in APP transgenic mice.

EXPERIMENTAL PROCEDURES

TrxAβ Constructs—Chimeric Trx polypeptides bearing the Aβ15 or the Aβ42 peptide (TrxAβ) were constructed by using a modified pET28 plasmid (Novagen), designated as pT7Kan-Trx, harboring the sequence for an N- and C-terminally His_6-tagged version of bacterial thioredoxin along with a kanamycin resistance marker. The unique CpoI site present within the Trx coding sequence (nucleotide positions 99–105, corresponding to amino acid residues 34–35) was used as the cloning site. The phosphorylated oligonucleotides 5′-gtccgatggatgcagaattccgacatgactcaggatatgaagttcatcatcaaggcg-3′ (forward) and 5′-gaccgccttgatgatgaatttcatatcctgagtcatgtcggaattctgcatccatcg-3′ (reverse), both bearing 5′-protruding CpoI sequences (underlined), were annealed and ligated to CpoI-digested pT7Kan-Trx at a 1:10 vector:insert molar ratio. The resulting construct, pT7Kan-TrxAβ15, encodes a polypeptide, Trx-(1–33)gPMDAEFRHDSGYEVHHQGGpTrx (36–109) in which Aβ15 (underlined) is preceded by a Met residue at the N terminus and is followed by the Gly-Gly-Pro linker at the C terminus (with the Gly and Pro residues provided by the inserted ds-oligonucleotide and by the Trx scaffold, respectively). Constructs bearing multiple copies of Aβ15 were generated in a similar way but at a 1:100 vector:insert molar ratio using the sequence-verified insert excised from pT7-Kan-TrxAβ15 as starting material. Recombinant clones were screened by restriction analysis, and two of them, bearing four or eight copies of Aβ15, were selected. Essentially identical experimental conditions were used to construct TrxAβ42, which was cloned in both pT7Kan-Trx as well as in a companion vector (pT7Amp-Trx) carrying an ampicillin rather than a kanamycin antibiotic resistance marker.

Expression and Purification of the TrxAβ Polypeptides—Expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside to *Escherichia coli* BL21Star (DE3) cells (Invitrogen) transformed with each of the above constructs and allowed to proceed for 2 h at 37 °C. A different *E. coli* strain (Origami-DE3; Novagen) and modified expression conditions (pT7Amp-Trx vector; 5 h at 30 °C) were used for TrxAβ42, which was otherwise completely insoluble. Following cell lysis, His_6-tagged TrxAβ polypeptides were bound to a metal affinity resin (Talon; Clontech), purified according to the manufacturer's instructions, and extensively dialyzed against phosphate-buffered saline (PBS). Protein concentration was determined with the Coomassie dye method (Bio-Rad) and by UV light absorbance. The composition and purity of individual polypeptide preparations was assessed by gel electrophoresis on 11% polyacrylamide-SDS gels.

Immunization Protocols—TrxAβ polypeptides (2 mg/ml in PBS) were filter-sterilized, and an aliquot of each (10 nmol) was mixed with 1 mg of alum (Sigma-Aldrich) in a final volume of 400 μl immediately before use. Aβ42 (Sigma-Aldrich) was dissolved in PBS (2 mg/ml) and aggregated overnight at 37 °C prior to immunization. Five randomly assorted groups of one-month-old male BALB/c mice (10 animals each) (Charles River Laboratories) were injected subcutaneously with the above antigens at days 1, 15, 30, and 60, as specified in the legend to Fig. 2*A*. The same treatment was applied to two negative control groups that were injected with PBS and with aggregated Aβ42, both without alum. Sera were collected two weeks after the last boost and randomly pooled in pairs. A standard immunization protocol (three doses of $Trx(Aβ15)_4$ antigen in Freund adjuvant administered to one animal over a period of two months) was used to generate rabbit anti-$Trx(Aβ15)_4$ antibodies (SeqLab).

Detection of Anti-Aβ42 Antibodies—Total anti-Aβ42 antibodies were detected by enzyme-linked immunosorbent assay (ELISA) at a fixed 1:200 dilution, using aggregated Aβ42 (0.5 μg/well) as the target antigen (43). Following incubation, washing, and the addition of horseradish peroxidase-conjugated anti-mouse immunoglobulins (1/5000; Sigma-Aldrich) and the chromogenic substrate *o*-phenylendiamine (Sigma-Aldrich), plates were read spectrophotometrically at 450 nm. Immunoglobulin isotype determination was conducted at a fixed 1:200 dilution using rat anti-mouse Ig subclass-specific, horseradish peroxidase-conjugated secondary antibodies (TechniPharm). ELISAs were conducted in triplicate on the five-paired sera from each group; only a subset of sera from the three top responders in Groups 3, 4, 6, and 7 (see Fig. 2*A*) was utilized for isotype determination. Comparisons between groups were conducted by one-way analysis of variance using Analyze-it software.

Immunohistochemistry—Sera from mice immunized with each of the three $Trx(Aβ15)_n$ polypeptides were screened for their ability to bind Aβ plaques in human brain sections from a 68-year-old patient with neuropathological and clinical symptoms typical of severe AD. Various dilutions (1:100–1:1000) of pooled sera from the three top responders in Groups 5, 6, and 7

were analyzed. Sera were added to serial 8-μm brain sections of formalin-fixed, temporal cortical tissue, pretreated with formic acid (80%, 15 min). Sera from mock-treated animals (PBS; Group 1) and a commercial anti-Aβ40 polyclonal antibody (Anti-Pan β-Amyloid, BIOSOURCE) were used as negative and positive controls, respectively. Immunolabeling was revealed with the EnVision Plus/horseradish peroxidase system (Dako), using 3–3′-diaminobenzidine as the chromogenic substrate, according to the manufacturer's instructions. Images were captured with a digital camera at magnifications ranging from 50 to 400×.

Immunodot Blot Assays and Atomic Force Microscopy Imaging—A stock solution of trifluoroacetic acid-pretreated, monomeric Aβ42 peptide (1 mM) was prepared as described previously (44) and diluted to the required Aβ42 final concentration (2–50 μM) with 0.1% trifluoroacetic acid prior to immunodot blot analysis. Aβ42 dissolved in 2 M dimethyl sulfoxide (1 mM final concentration) was utilized for the preparation of aggregated Aβ42 species (19, 21, 45). A 10-fold dilution of the above stock solution into cold Ham's F-12 K medium (phenol red-free; BIOSOURCE) followed by incubation at 4 °C for 24 h was used to produce soluble oligomers; the same stock solution diluted into 10 mM HCl at a final concentration of 100 μM and incubated for 24 h at 37 °C was used to generate Aβ fibrils. Aβ oligomer and fibril formation, as well as the absence of fibrillar aggregates in soluble oligomer preparations, were verified by atomic force microscopy. To this end, samples of the above-described Aβ42 preparations were diluted 10-fold in 20 μl of deposition buffer (4 mM HEPES, pH 7.4, 10 mM NaCl, 7 mM $MgCl_2$) and immediately deposited onto freshly cleaved ruby mica at room temperature. After 5 min, mica disks were rinsed with milli-Q grade water and gently dried under a stream of nitrogen. Images were collected with a Nanoscope III microscope (Digital Instruments) operated in tapping mode using commercial diving board silicon cantilevers (MikroMasch). Oligomer formation was also checked by electrophoretic analysis on Tris-Tricine polyacrylamide gels followed by silver staining as described in Ref. 19. Samples of recombinant $Ile^{84} \rightarrow Ser$ transthyretin (Ser^{84}-TTR) enriched in soluble oligomers or higher order fibrillar aggregates were prepared by incubating the protein (2 mg/ml) for either 2 or 96 h at 37 °C in 100 mM sodium-acetate buffer (pH 4.0) as described in Refs. 46 and 47; control samples of monomeric Ser^{84}-TTR were obtained from parallel incubations in 50 mM K-phosphate buffer (pH 7.6). For dot blot analysis, the various Aβ42 or Ser^{84}-TTR species were hand-spotted onto nitrocellulose membranes (GE Healthcare) pre-wetted with TBS (20 mM Tris-HCl, pH 7.5, 0.8% NaCl) (19). Antisera for dot blot analysis were affinity-purified on protein-A minicolumns (Diatheva) according to the manufacturer's instructions. Following the determination of total immunoglobulin concentration with the Coomassie dye method, purified immunoglobulins were used for dot blot assays at a final concentration of 0.75 μg/ml. After blocking at room temperature with 5% nonfat dry milk in TBS supplemented with 0.05% Tween 20 (TBST), the blots were incubated for 1.5 h with each of the three anti-$Trx(A\beta15)_n$ antibodies in dry milk-TBST, washed 3 × 10 min with TBST, followed by mouse immunoglobulin detection with the SuperSignal West Femto kit (Pierce), as specified by the manufacturer. To distinguish between monomer and oligomer binding, affinity-purified anti-monomeric $TrxA\beta15$ antibodies (0.75 μg/ml) were preincubated (2 h at 4 °C under stirring conditions) with a 50-fold molar excess of the monomeric Aβ42 peptide in TBS prior to hybridization. At least two replicates were carried out for each of the 15 pools of antisera from the various $Trx(A\beta15)_n$-treated groups (see Fig. 2*A*, *Groups 5–7*). Dot blot images were quantified with the Quantity One program (Bio-Rad). Signal volume intensities for individual Aβ42 species within each membrane were averaged, and the resulting data were plotted with Sigma Plot (SPSS).

Surgery and Neuropathological Evaluation of Transgenic AD Mice—Female transgenic AD (Tg2576) mice expressing the Swedish mutation of human APP (48) were obtained from the Boston University Alzheimer Disease Center mouse colony. Founders for this colony were provided by Dr. Karen Hsiao-Ashe (Department of Neurology, University of Minnesota Medical School). The APP Tg2576 mice developed behavioral abnormalities and exhibited histological evidence of brain Aβ deposits as plaques along with associated astrogliosis from as early as 8 months. Mice were genotyped using a standardized PCR assay on tail DNA and were housed four in each cage under standard conditions with *ad libitum* access to food and water. Six 14-month-old APP mice (32–34 g each), placed on a 12-h light schedule, were used for surgeries. Mice were anesthetized with ketamine HCl/xylazine intraperitoneal injection (100 mg/kg ketamine and 10 mg/kg xylazine; 100 μl/10 g body weight) and were positioned in a stereotaxic apparatus (Koph) with a mouse head adaptor. Thermoregulation was maintained at 37 °C using a warming pad with respiratory monitoring throughout the procedure. The scalp was incised in the midline to expose the sagittal suture, and stereotaxic coordinates in both hemispheres were determined (49). The bregma was used as a reference point (−2.0 mm), and holes were drilled in the calvarium at the junction of the left and right lateral coordinates (1.75 mm). Affinity-purified anti-$Trx(A\beta15)_4$ antibodies along with mock immunoglobulins from PBS-treated mice (2 μl each) were stereotaxically injected into the left and right hippocampi (−2.0 mm ventral), respectively, using a blunt-tipped 10-μl syringe (Hamilton). Upon syringe placement, there was a 2-min dwell time followed by a 4-min injection time and an additional 2-min dwell time prior to removal of the syringe. A topical antiseptic was applied as the incision was closed using a 9-mm autoclip. Mice were kept on a warming pad until full recovery. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and both the Department of Veterans Affairs and Boston University Animal Care committees. Seven days post-injection, the mice were deeply anesthetized and transcardially perfused with 2% buffered paraformaldehyde (100 ml). Brains were post-fixed for 2 h, cryoprotected in a graded series of glycerol, and subsequently frozen-sectioned (50 μm). Serially cut mouse tissue sections were stained for Nissl substance, immunostained with anti-Aβ42 (catalog number 344; BIOSOURCE International), anti-Aβ oligomer (A11; BIOSOURCE International), and glial fibrillary antigen protein (Dako) antibodies and silver-stained

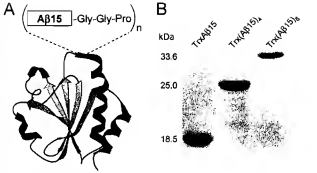

FIGURE 1. **Construction of Trx(Aβ15)$_n$ chimeras.** *A*, schematic representation of Trx-displayed Aβ15 peptides with their associated tripeptide linker. *B*, SDS-PAGE analysis of the purified Trx(Aβ15)$_n$ polypeptides.

using the Campbell-Switzer method for identification of mature Aβ plaques. Immunostained coronal tissue sections, serially cut within the hippocampus beginning from interaural (1.68 mm)/bregma (−2.12 mm) to interaural (2.16 mm)/bregma (−1.64 mm), were analyzed. Aβ-positive plaques were quantified from high resolution images of the same brain areas within the anti-Trx(Aβ15)$_4$-treated hemisphere and the contralateral mock-treated hemisphere using BioVision (50) and Neurolucida software programs (MicroBrightField, Williston, VT). BioVision differentiates and counts plaques from the background neuropil, whereas Neurolucida extracts the data from the BioVision images, exporting it to Excel (Microsoft, Redmond, WA) for statistical analysis.

RESULTS

Design and Construction of the Trx(Aβ15)$_n$ Polypeptides—The choice of Aβ15 as a peptide epitope was based on the lack of T-cell reactivity and good solubility previously ascribed to N-terminal peptides encompassing the first 15 residues of Aβ (4, 14, 27, 30, 43, 51). Trx was chosen because of its small size (109 amino acids) and solubility, structural rigidity, ability to act as a nontoxic immunoenhancer capable of stimulating murine T-cell proliferation, and presence of a convenient restriction site (CpoI) within its solvent-accessible active site loop (40–42). Insertions into this loop are usually well tolerated, and grafting both ends of the inserted peptide provides a conformational constraint that are neither achievable with N- or C-terminal fusions nor with random chemical cross-linking of peptides to a protein carrier (38–40). To mimic different assembly states of Aβ and to overcome the poor immunogenicity of short Aβ fragments (25, 27), Trx(Aβ15)$_n$ polypeptides bearing a single copy or multiple tandemly arranged copies of Aβ15 were constructed. A cloning strategy relying on the use of an excess of the Aβ15 DNA insert with respect to a modified recipient vector bearing the Trx coding sequence under the control of a phage T7 promoter (see "Experimental Procedures") was utilized for Trx(Aβ15)$_n$ construction (Fig. 1*A*). Constructs bearing one, four, or eight copies of Aβ15 were used to express the corresponding polypeptides, which were then purified by metal affinity chromatography (Fig. 1*B*). Instrumental to the production of properly assembled Aβ15 multimers were the directionality and in-frame fusion capability of the unique CpoI site present within the Trx active site loop region as well as the incorporation into Aβ15 DNA of a terminal sequence coding for an intervening Gly-Gly-Pro linker, thus also preventing the formation of junctional epitopes (52). A fourth construct (TrxAβ42) bearing a single copy of the full-length Aβ42 peptide was prepared in a similar way (see "Experimental Procedures" for details). Although all Trx(Aβ15)$_n$ polypeptides were completely soluble, regardless of Aβ15 multiplicity, most of the TrxAβ42 protein ended up in inclusion bodies in an insoluble form (not shown). Thus, Aβ42 appears to be poorly soluble, even when fused to Trx in the heterologous context of bacterial cells.

Immunological Characterization of the Trx(Aβ15)$_n$ Polypeptides—Five groups of 10 male BALB/c mice were treated with 10 nmol of the above-described Trx(Aβ15)$_n$ polypeptides or with equivalent amounts of preaggregated synthetic Aβ42 or TrxAβ42, all supplemented with alum, an immunoadjuvant approved for human use (Fig. 2*A*). Two additional groups injected with buffer alone (PBS) or with alum-free Aβ42 served as negative controls. No adverse side effect was observed in animals injected with four doses of the various Trx(Aβ15)$_n$ polypeptides over a period of two months. Sera were collected two weeks after the fourth injection and randomly pooled in pairs, and the five resulting pools were analyzed with ELISA using aggregated fibrillar Aβ42 as the target antigen. As shown in Fig. 2*A*, mean anti-Aβ antibody levels elicited by Trx(Aβ15)$_4$ and Trx(Aβ15)$_8$ (but not monomeric TrxAβ15) were significantly higher ($p \leq 0.05$) than those of mock-treated controls and similar to those of the Aβ42-treated groups, where TrxAβ42 performed as well as free Aβ42. An anti-inflammatory T helper 2-polarized response, typical of the alum adjuvant (14, 25, 53), was revealed by isotype profiling (Fig. 2*B*). Although a prevalence of IgG1 was observed with all antigens, the IgG1:IgG2a ratio, an indicator of T helper 2 polarization, was reproducibly higher ($p \leq 0.05$) for multimeric Trx(Aβ15)$_n$ and TrxAβ42 immunoconjugates than for unconjugated Aβ42.

Detection of Aβ Aggregates in Human Brain by Anti-Trx(Aβ15)$_n$ Antibodies—The ability of antisera generated in response to Trx(Aβ15)$_n$ to bind amyloid plaques was investigated next. This property, which is considered the best prognostic indication of *in vivo* anti-Aβ antibody efficacy (26), is not shared by all previously described anti-Aβ antibodies (*e.g.* m266 and other antibodies targeting the C-terminal portion of Aβ42 (26)). As shown in Fig. 3, sera from mice immunized with the tetrameric (Fig. 3*A*) or the octameric (Fig. 3*B*) form of Trx(Aβ15)$_n$ bound to amyloid plaques up to a dilution of 1:1000. Large neuritic plaques, as well as mature and immature plaques, were labeled by anti-multimeric Trx(Aβ15)$_n$ antibodies. A broader immunostaining, especially within senile plaque cores, was observed with the positive control anti-Pan β-amyloid antiserum generated in rabbits using Aβ40 as the antigen (not shown). By comparison, no plaques were detected either with sera from mock-treated animals (not shown) or with sera from mice immunized with monomeric TrxAβ15 (Fig. 3*C*).

Differential Recognition of Distinct Aβ Assembly States by Anti-Trx(Aβ15)$_n$ Antibodies—Immunodot blot assays were used then to assess the recognition selectivity of the various anti-Trx(Aβ15)$_n$ antibodies toward different assembly states of

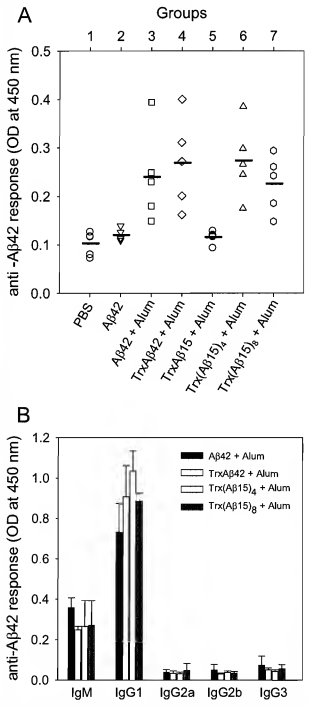

FIGURE 2. **Immunological characterization of Trx(Aβ15)$_n$ polypeptides.** *A*, anti-Aβ42 reactivity of sera from mice immunized with the indicated Trx(Aβ15)$_n$ polypeptides (*Groups 5–7*), with preaggregated synthetic Aβ42 (*Group 3*), or TrxAβ42 (*Group 4*), all adjuvanted with alum, or mock-immunized with either adjuvant-free Aβ42 (*Group 2*) or PBS (*Group 1*) (see "Experimental Procedures" for details). ELISA data for five randomly paired pools of sera from each group were obtained using aggregated synthetic Aβ42 as target antigen and are presented as a scatter plot; the Aβ binding activity of the 35 individual pools as well as the mean binding activity of each of the seven groups (*bars*) are shown. *B*, Immunoglobulin isotypes of antisera from the three top responders in each immunopositive group (*3*, *4*, *6*, and *7*).

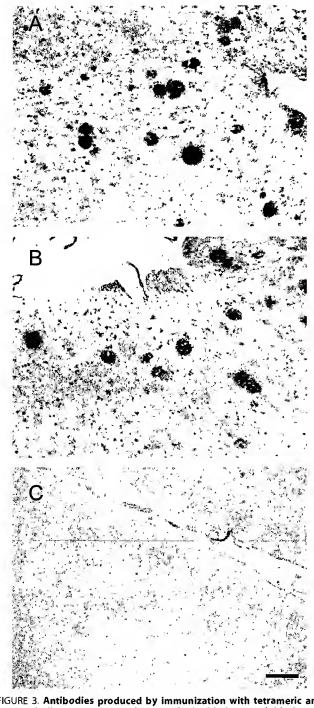

FIGURE 3. **Antibodies produced by immunization with tetrameric and octameric (but not monomeric) Trx(Aβ15)$_n$ bind to amyloid plaques (*dark brown spots*) in the brain.** Temporal cortex sections from a patient with severe AD were treated with sera from mice immunized with Trx(Aβ15)$_4$ (*A*), Trx(Aβ15)$_8$ (*B*), or TrxAβ15 (*C*). *Scale bar*, 100 μm.

Aβ42 (monomers, oligomers, and fibrils) generated *in vitro* under previously determined conditions (21, 45). Fibril formation, the lack of Aβ aggregates in the monomer solution, as well as the lack of fibrils in the oligomer preparation were verified by atomic force microscopy (Fig. 4*A*). Silver-stained polyacrylamide gels were used to evaluate oligomer preparations, which in keeping with previous reports (19, 21, 45) and despite prolonged incubation in F-12 medium, were found to contain ~50% residual monomer, with trimers and tetramers as the most represented oligomeric species (Fig. 4*B*). As shown in Fig. 4*C*, antibodies from mice immunized with monomeric

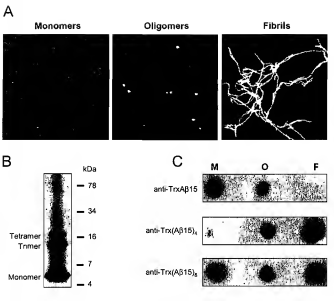

FIGURE 4. **Differential recognition of distinct Aβ assembly states by antibodies raised against different Trx(Aβ15)$_n$ polypeptides.** *A*, representative 2 × 2 μm atomic force microscopy images of the indicated Aβ42 species; total *z*-ranges (*left to right*) are 2, 12, and 13 nm. *B*, silver-stained SDS-PAGE of a typical Aβ42 oligomer preparation; the migration positions of individual Aβ42 species and of molecular mass markers are indicated. *C*, representative results obtained from immunodot blot assays carried out at a fixed concentration (0.75 μg/ml) of the indicated affinity-purified anti-Trx(Aβ15)$_n$ antibodies and 10 pmol of the various Aβ42 species (*M*, monomers; *O*, oligomers; *F*, fibrils). *D*, differential recognition profiles of antibodies from the five pools of antisera in each of the Trx(Aβ15)$_n$-treated groups (Group 5, anti-Trx(Aβ15); Group 6, anti-Trx(Aβ15)$_4$; Group 7, anti-Trx(Aβ15)$_8$; see Fig. 2*A*). Dot blot assays (at least two replicates for each pool) were conducted as described for *C* and quantified; signal volume intensities for individual Aβ42 species within each membrane were averaged. Cumulative data for the three groups are expressed as relative values (± S.D.) for each Aβ42 species, normalized with respect to the sum of the average hybridization signal intensities measured for all Aβ42 species.

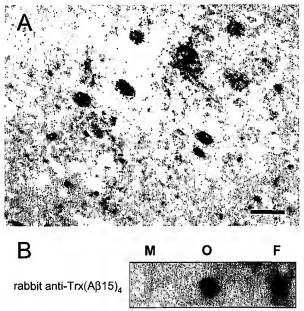

FIGURE 5. **Aβ recognition properties of a rabbit anti-Trx(Aβ15)$_4$ antiserum obtained with Freund's adjuvant.** *A*, immunostaining of amyloid plaques (*dork brown spots*) in human brain. *Scale bar*, 100 μm. *B*, immunodot blot assay of different Aβ42 species (*M*, monomers; *O*, oligomers; *F*, fibrils (10 pmol each)).

TrxAβ15 do not bind to Aβ fibrils, in accordance with their inability to recognize higher order Aβ42 aggregates in ELISAs or Aβ fibrils in AD plaques (see Figs. 2*A* and 3*C*), yet they appear to react with both monomers and oligomers. However, because of the presence of sizeable amounts of monomeric Aβ in the oligomer preparation (see Fig. 4*B*), it is likely that Aβ monomers, rather than oligomers, are the actual targets of anti-monomeric TrxAβ15 antibodies. In fact, a loss of immunoreactivity was observed upon preincubation of these antibodies with an excess of the monomeric Aβ42 peptide (not shown). A different recognition pattern was observed with antibodies raised against Trx(Aβ15)$_4$, which recognized Aβ42 oligomers and fibrils but not monomers (Fig. 4*C*). In contrast but similar to the results obtained with the commercial 4G8 monoclonal antibody (not shown), all three Aβ42 species were bound by antibodies raised against Trx(Aβ15)$_8$ (Fig. 4*C*), thus indicating that excessive multimerization of the Aβ15 peptide leads to a loss of conformational selectivity. This also appears to be the case for a recently developed DNA immunogen composed of an unscaffolded 11-fold repeat of Aβ1–6 without an intervening spacer, which yielded antibodies that indiscriminately recognized monomeric and oligomeric Aβ42 species yet barely bound to higher order fibrillar aggregates (28). Importantly, as shown in Fig. 4*D*, the same differential recognition profile was shared by the different, randomly assorted pools of antisera raised against monomeric, tetrameric and octameric TrxAβ15. As further shown in Fig. 5, the same conformational selectivity along with the capacity to recognize human Aβ plaques were observed with a rabbit antiserum generated using Freund-adjuvanted Trx(Aβ15)$_4$ as antigen, thus suggesting that this property is neither influenced by the animal host nor the type of adjuvant utilized for immunization.

Additional evidence as to the conformational selectivity of anti-Trx(Aβ15)$_4$ antibodies was provided by an immunodot blot experiment testing their ability to recognize different assembly states of an amyloidogenic polypeptide unrelated in sequence to Aβ42. The Ile^{84} → Ser variant of human transthyretin, a strongly amyloidogenic protein (54), was used for this purpose. As shown in Fig. 6, although with a reduced immunoreactivity, the same differential recognition pattern observed with Aβ42 (*i.e.* the binding of higher order fibrillar aggregates and oligomers but not monomers) was observed with anti-Trx(Aβ15)$_4$ antibodies when challenged with different assembly states of Ser^{84}-TTR. As expected, no reactivity toward aggregated Ser^{84}-TTR was observed under the same conditions with antibodies raised against monomeric TrxAβ15 (not

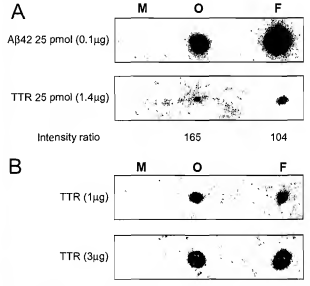

FIGURE 6. **Binding of anti-Trx(Aβ15)$_4$ antibodies to aggregated (but not monomeric) Ser^{84}-TTR.** *A*, affinity-purified anti-Trx(Aβ15)$_4$ antibodies (0.75 μg/ml) were comparatively analyzed for their ability to recognize distinct assembly states of Aβ42 and Ser^{84}-TTR (*M*, monomers; *O*, oligomers; *F*, fibrillar aggregates). The ratios between hybridization values for Aβ42 and Ser^{84}-TTR, obtained by densitometric analysis after 1 min of signal development, are shown *below* the *O* and *F rows*. *B*, binding of oligomeric and fibrillar Ser^{84}-TTR by anti-Trx(Aβ15)$_4$ antibodies visualized in an immunodot blot experiment carried out as described for *A* but with a longer signal development time (15 min).

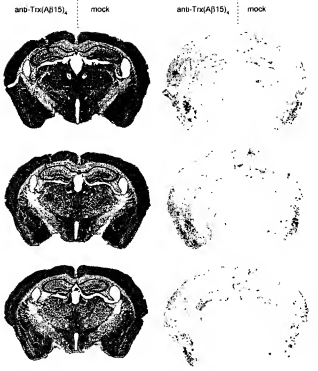

FIGURE 7. **Gross identification of stereotaxic injection sites in the APP Tg2576 mice.** Serially cut Nissl-stained sections and matching contiguous Aβ42-immunostained sections are shown on the *left* and *right* sides, respectively. Affinity-purified anti-Trx(Aβ15)$_4$ antibodies, along with mock immunoglobulins from PBS-treated mice (2 μl each), were stereotaxically injected into the left and right hippocampus as indicated; needle tracts are marked by *asterisks*. Tissue sections, 1 and 2 mm rostral to the injection sites, are shown in *B* and *C*, respectively. Adjacent immunostained sections in *A–C* show a marked reduction of Aβ42-positive plaques close to the anti-Trx(Aβ15)$_4$ injection site and penumbra. *Scale bar*, 2 mm.

shown), thus further strengthening the notion that these antibodies selectively recognize Aβ monomers.

Anti-Trx(Aβ15)$_4$ Antibody Clears Aβ Pathology in APP Transgenic AD Mice—The immunotherapeutic potential of anti-Trx(Aβ15)$_4$ was evaluated next by stereotaxically injecting this antibody into the hippocampus of 14-month-old APP transgenic AD (Tg2576) mice. Mock immunoglobulins injected into the contralateral hemisphere from mice treated with PBS only served as an internal control for this experiment. Seven days post-injection, histopathological examination and plaque quantitation revealed a significant ($p < 0.01$) reduction of Aβ immunostaining in the anti-Trx(Aβ15)$_4$-injected hemispheres ($0.97 \times 10^3 \pm 0.27$ plaques) compared with the mock-injected hemispheres ($3.34 \times 10^3 \pm 0.58$ plaques) (Figs. 7 and 8, *A–C*). Aβ-positive plaques were not only absent at the injection site but significantly diminished within the injection penumbra (2 mm anterior/posterior to the injection site) (Figs. 7 and 8, *A–C*). As shown in Fig. 8*D*, similar results were obtained with an antibody that selectively reacts with soluble high molecular weight Aβ40 and Aβ42 oligomers (36). This indicates that the clearing effect of anti-Trx(Aβ15)$_4$ does not depend on the particular primary antibody utilized for immunodetection and suggests that also higher order oligomers (23) are targeted *in vivo* by the anti-Trx(Aβ15)$_4$ antibody. To verify that these findings were not the result of a competition between anti-Trx(Aβ15)$_4$ and the primary anti-Aβ antibody, we performed alternative histopathological analyses using glial fibrillary antigen protein immunostaining and Campbell-Switzer silver staining to detect astrogliosis and Aβ plaques. An amyloid plaque-associated astroglial response, increasing with the severity of disease progression, has been reported in APP mice (55). As shown in Fig. 9, *A* and *B*, astrogliosis and glia-associated plaques were markedly reduced within the anti-Trx(Aβ15)$_4$ antibody injection penumbra compared with the contralateral mock-injected hemisphere. In addition, as revealed by Campbell-Switzer silver staining, there were far fewer plaques in the anti-Trx(Aβ15)$_4$-injected hemisphere compared with the mock-injected hemisphere (*cf.* the *left* and the *right panels* in Fig. 9*C*). Both observations are consistent with the immunostaining data obtained with anti-Aβ antibody detection.

DISCUSSION

As revealed by this study, Trx(Aβ15)$_4$ is a soluble derivative of Aβ with good immunogenicity even when formulated with a moderate strength adjuvant, such as alum, that promotes an anti-inflammatory T helper 2-polarized immunoresponse. Even more significant, Trx(Aβ15)$_4$ is the first B-cell epitope Aβ antigen that is shown to be capable of generating antibodies that bind to Aβ fibrils and synaptotoxic Aβ oligomers but not to the presumably physiological monomeric species of Aβ. In this regard, anti-Trx(Aβ15)$_4$ antibodies resemble assembly state selective antibodies previously developed by using preassembled Aβ42 oligomers (19) or the nitrated Aβ40 peptide (35) as antigens. The main difference here is that a selected Aβ fragment, rather than full-length Aβ, was used as a recombinant

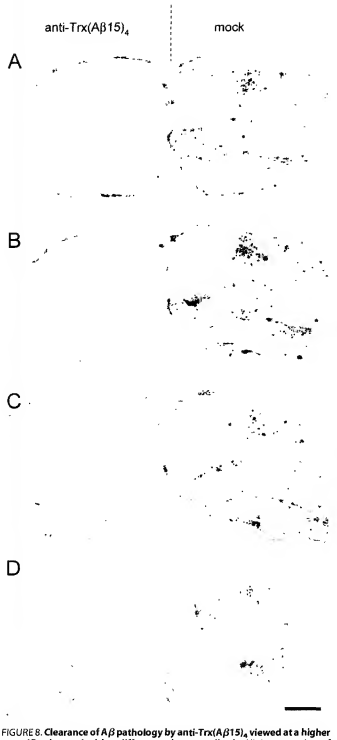

FIGURE 8. **Clearance of Aβ pathology by anti-Trx(Aβ15)$_4$ viewed at a higher magnification and with a different primary antibody.** High power view of brain sections from Tg2576 mice stereotaxically injected as described in the legend to Fig. 7 (anti-Trx(Aβ15)$_4$, left hemisphere; mock, right hemisphere), immunostained with either a universal anti-Aβ42 antibody (*A–C*) or an anti-oligomer-selective antibody (*D*) (see "Experimental Procedures" for details). Sections corresponding to the injection site or to the penumbra, shown in *A* and in *B–D*, respectively, evidence the lack of Aβ-positive plaques in the hippocampus and overlying cortex of the anti-Trx(Aβ15)$_4$-injected hemisphere. *Scale bar*, 500 μm.

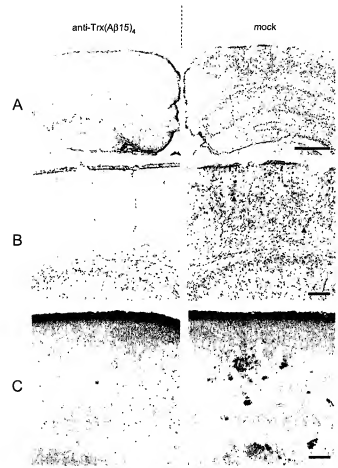

FIGURE 9. **Anti-Trx(Aβ15)$_4$ treatment reduces astrogliosis as well as the number of silver-positive plaque structures.** Tissue sections from stereotaxically injected Tg2576 mice (anti-Trx(Aβ15)$_4$, *left side*; mock, *right side*) were either immunostained with an antibody against glial fibrillary antigen protein, a histological marker of astrogliosis (*panels A* and *B*), or silver-stained with the Campbell-Switzer method (*C*). At low (*A*; *scale bar*, 500 μm) and high (*B*; *scale bar*, 100 μm) magnification, glial fibrillary antigen protein immunostaining reveals little or no reactive astrogliosis and glia-associated plaques (*arrowheads* in *B*) in the anti-Trx(Aβ15)$_4$-injected side (*left*) in contrast to the mock-injected side (*right*). A similar effect of the anti-Trx(Aβ15)$_4$ antibody on plaque burden, detected with Campbell-Switzer silver staining, is shown in *C*.

antigen and that antibodies with differential recognition properties were generated simply by varying the copy number of the Aβ15 peptide within the display site of thioredoxin. The ability of anti-Trx(Aβ15)$_4$ antibodies to recognize oligomeric and fibrillar aggregates of a different amyloidogenic protein such as TTR, is reminiscent of the "pan-amyloid reactivity" previously observed with antibodies raised against sonicated Aβ40 fibrils (34) or Aβ40 immobilized on gold nanoparticles (36) as structurally constrained antigens. Various hypotheses have been proposed to explain this cross-reactivity. For example, regardless of their amino acid sequence, all amyloids may share a cross-β pattern structure or other common features, such as a unique array of H-bond donors and acceptors (34). Apart from the, as yet, largely unknown structural determinants of this cross-reactivity, what our data suggest is that, when grafted to the active site loop of thioredoxin, the (Aβ15)$_4$ polypeptide is constrained into a conformation that mimics a structural epitope shared by both fibrils and neurotoxic soluble oligomers but not Aβ monomers. Conformational selectivity, coupled with enhanced immunogenicity, may increase the safety and efficacy of anti-Trx(Aβ15)$_4$ as an Aβ aggregate scavenger. This

expectation is supported by the immunotherapeutic performance of the anti-Trx(Aβ15)$_4$ antibodies, revealed by the *in vivo* experiments in APP transgenic AD (Tg2576) mice. These mice recapitulate many aspects of human AD and provide a means to investigate the temporal and spatial progression of several important neuropathological events, such as amyloid deposition and astrogliosis. Our experimental studies show significant amelioration of the neuropathological phenotype displayed by this AD mouse model following administration of the anti-Trx(Aβ15)$_4$ antibody.

Because of the above-mentioned favorable features, Trx(Aβ15)$_4$ lends itself as a prototype B-cell epitope vaccine for A.D. Because of its recombinant nature and chemically defined molecular composition, Trx(Aβ15)$_4$ is also a promising candidate for a DNA vaccination approach for the production of monoclonal antibodies for passive immunization purposes as well as for structural analysis of its amyloid-like epitope.

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Exhibit 4

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(54) **MONOCLONAL ANTIBODIES SPECIFIC FOR HIGH MOLECULAR WEIGHT AGGREGATION INTERMEDIATES COMMON TO AMYLOIDS FORMED FROM PROTEINS OF DIFFERING SEQUENCE**

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(57) **ABSTRACT**

Methods for the production of monoclonal antibodies specific to conformational epitope(s) of a prefibrilar aggregate(s) which contribute to amyloid fibril formation in human or animal subjects who suffer from amyloid diseases (e.g. Alzheimer's Disease) and the hybridomas and monoclonal antibodies produced therefrom. Also, the use of such monoclonal antibodies in the immunization of human or animal subjects against Alzheimer's Disease or other amyloid diseases and/or for the diagnosis or detection of Alzheimer's Disease or other amyloid diseases. The monoclonal antibodies may be administered concomitantly or in combination with anti-inflammatory agents, such as gold or gold containing compounds, to decrease neural inflammation associated with amyloid diseases (e.g. Alzheimer's Disease).

Fig. 1

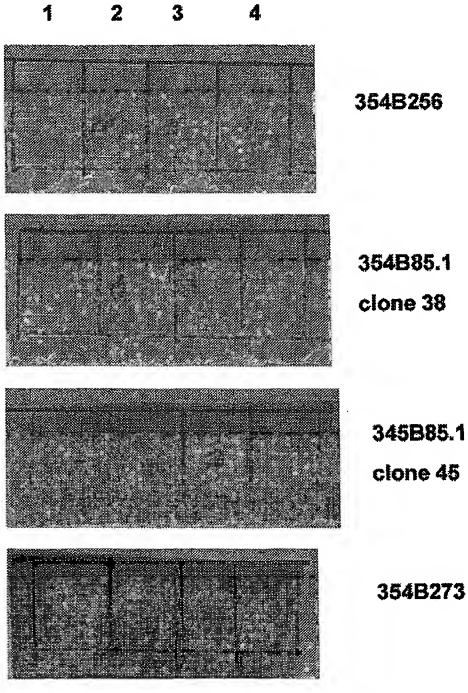
Fusion ID 354B
Mouse # 1867/11 #5684

Hybrid	ELISA*			Note	Frozen	Cloning
	Sol	Fibril	Interm			
354B67	0.145	0.068	0.102	High Titer	7/2/03	Not Cloned
354B85	0.139	0.179	0.183	High Titer	7/2/03	6/26/03
354B133	0.102	0.084	0.069	High Titer	7/4/03	6/26/03
354B162	0.216	0.237	0.291	High Titer	7/2/03	6/26/03
354B256	0.056	0.086	0.367	Specific	7/21/03	6/26/03
354B273	0.320	0.292	0.325	Specific	7/10/03	6/27/03

*Low ELISA titers were attributed to older coated plates

Clone	ELISA		Note	Frozen
	Sol	Interm		
354B85#44	0.059	0.181	Good results	8/5/03
354B85#45	0.074	0.274	Good results	8/5/03
354B85#52	0.042	0.492		
354B85#56	0.065	0.159	Good results	8/18/03
354B85#11			Good results	8/5/03
354B85#18			Good results	8/5/03
354B85#38			Good results	8/5/03
354B133#31	0.041	0.733		
354B162#14	0.033	0.582		
354B162#24	0.041	1.030		
354B162#32	0.045	0.545		
354B162#63	0.036	0.928		
354B162#64	0.038	1.104	Good results	8/5/03
354B162#42			Picked	8/12/03
354B162#43				
354B162#44				
354B162#46				
354B162#47				
354B162#48				
354B162#57				
354B256#17	0.042	0.244		
354B256#31	0.029	0.807		
354B256#15				
354B256#46				
354B256#55			Picked	8/14/03
354B273#11	0.521	0.507		
354B273#15	0.436	0.537		
354B273#25	0.503	0.533		
354B273#33	0.469	0.512		
354B273#13				
354B273#14				
354B273#31			Best signal	8/14/03

Fig. 2



MONOCLONAL ANTIBODIES SPECIFIC FOR HIGH MOLECULAR WEIGHT AGGREGATION INTERMEDIATES COMMON TO AMYLOIDS FORMED FROM PROTEINS OF DIFFERING SEQUENCE

RELATED APPLICATION

[0001] This patent application claims priority to U.S. Provisional Patent Application No. 60/502,326 filed on Sep. 12, 2003, the entirety of which is expressly incorporated herein by reference.

FIELD OF THE INVENTION

[0002] This invention relates generally to the fields of medicine, immunology and protein biochemistry and more particularly to a) methods for the production of monoclonal antibodies specific to conformatonal epitope(s) of a pre-fibrillar aggregate(s) which contribute to amyloid fibril formation in human or animal subjects, b) the hybridomas and monoclonal antibodies produced therefrom, c) the use of such monoclonal antibodies in the immunization of human or animal subjects against Alzhiemer's Disease or other amyloid diseases and d) the use of such monoclonal antibodies in the diagnosis or detection of Alzhiemer's Disease or other amyloid diseases in human or animal subjects.

BACKGROUND OF THE INVENTION

[0003] Many biological functions come about, at least in part, due to the ability of proteins to adopt various sequence-dependent structures. However, certain protein sequences can sometimes form aberrant, misfolded, insoluble aggregates known as amyloid fibrils. These amyloid fibrils are thought to be involved in the pathogenesis of various amyloid diseases of genetic, infectious and/or spontaneous origin, including spongiform encephalopathies, Alzheimer's disease, Parkinson's disease, type II diabetes, Creutzfeldt-Jakob disease, Huntington's disease, possibly macular degeneration, various prion diseases and numerous others. In at least some of these amyloid diseases, amyloid fibrils lead to the development of amyloid plaques.

[0004] Amyloid peptides are the principal constituent of amyloid plaques. In the case of Alzheimer's disease, the peptides are termed A(3 or (3-amyloid peptide. A peptide is an internal fragment of 39 to 43 amino acids of amyloid precursor protein (APP). Several mutations within the APP protein have been correlated with the presence of AD. See, for example, Goate et al., Nature, (1991) 349, 704 (valine to isokucine); Chartier Marian et al., Nature (1991)353,844 (valine to glycine); Murrell et al. Science (1991) 21,97 (valine to phenylalanine); Mullan et al., Nature Genet. (1992) 1,345 (a double mutation changing lysine 595-methionine596 to asparagine595-leucine596). Such mutations are thought to cause AD by producing an increased or altered processing of APP to Aβ. In particular, the processing of APP resulting in accumulation of the longer forms of Aβ, for example, Aβ1-42 and Aβ1-43 is thought to be important in the cause of AD. Mutations in other genes, such as the presenilin genes PS1 and PS2, are thought to indirectly affect processing of APP resulting in production of the long form of Aβ. See, for example, Hardy, TINS (1997) 20,11.

[0005] It is believed that cytotoxic amyloid-beta peptide aggregates disrupt the integrity of cell membranes and elaborate reactive oxygen intermediates, thereby giving rise to elevations in cytosolic calcium and eventual cell death. Cell surface receptors for amyloid-beta peptide may also activate signal transduction mechanisms.

[0006] European Patent Publication EP 526,511 (McMichael) and PCT International Patent Publication WO/9927944 (Schenk) have described the administration of Aβ to patients for the treatment or prevention of Alzheimer's. However, although active immunization of Aβ to transgenic mice produces apparent benefits, the extension of this approach to AD patients has resulted in undesirable inflammation of the central nervous system in some of the subjects. See Hardy, D. J. Selkoe (2002) Science 297, 353-356. Soluble Aβ includes Aβ monomers as well as aggregations of such monomers referred to as prefibrillar aggregates. These prefibrillar aggregates lead to the development of amyloid fibrils.

[0007] Soluble Aβ content of the human brain is better correlated with the severity of AD than is the accumulation of amyloid plaques. See, for example, Y. M. Kuo et al. (1996) J. Biol. Chem. 271, 4077-4081; C. A. McLean et al. (1999) Annals of Neurology 46, 860-6; L. F. Lue et al. (1999) American Journal of Pathology 155, 853-862. In addition, recent reports suggest that the toxicity of A and other amyloidogenic proteins lies not in the soluble monomers or insoluble fibrils that accumulate, but rather in the prefibrillarprefibrillar aggregates. See, for example, Hartley et al. (1999), Journal of Neuroscience 19, 8876-8884; Lambert et al., Proceedings of the National Academy of Sciences of the United States of America (1998) 95, 6448-53; and Bucciantini et al., Nature (2002) 416, 507-511; and Hartley et al. Nature (2002) 418, 291. Taken together, these results indicate that the prefibrillar aggregates may be more pathologically significant than other forms of the amyloid peptides and therefore may be a more desirable target in the prevention or curing of amyloid diseases such as AD.

[0008] PCT International Patent Application PCT/US2003/028829 (WO 2004/024090) entitled MONOCLONAL ANTIBODYS AND CORRESPONDING ANTIBODIES SPECIFIC FOR HIGH MOLECULAR WEIGHT AGGREGATION INTERMEDIATES COMMON TO AMYLOIDS PORMED FROM PROTEINS OFDIFFERING SEQUENCE (Kayed and Glabe) describes compositions of matter comprising one or more conformational epitopes found on amyloid peptide aggregates, antibodies to such epitopes and methods for making and using the compositions, epitopes and/or antibodies. The compositions described in PCT/US2003/028829 include synthetic or isolated compositions that contain or consist of certain conformational epitopes found on peptide aggregates (e.g., toxic peptide aggregates) present in human or veterinary patients who suffer from, or who are likely to develop, amyloid diseases (e.g., Alzheimers Disease). The invention described in PCT/US2003/028829 also includes methods for using such compositions in the detection, treatment and prevention of diseases in humans or animals and/or in the testing and identification of potential therapies (e.g., drug screening) using such antibodies. The entirety of PCT International Patent Application PCT/US2003/028829 is expressly incorporated herein by reference.

[0009] Monoclonal antibodies are homogeneous preparations of immunoglobulin proteins that specifically recognize

and bind to regions, or epitones, of their corresponding antigens. In some cases, monoclonal antibodies can bind to and inhibit the activity of endogenous chemical entities that are toxic or deleterious. In view of this, there is a need for the development of new monoclonal antibodies that bind to and inhibit toxic forms of amyloid (e.g., cytotoxic amyloid-beta peptide aggregates or protofibrils) with high specificity, thereby providing for diagnosis and treatment of amyloid diseases.

SUMMARY OF THE INVENTION

[0010] The present invention provides compositions comprising isolated monoclonal antibodies which bind to one or more conformational epitope(s) of prefibrillar aggregate(s) that contribute to amyloid fibril formation in the brains of humans or animals (e.g., toxic species of prefibrillar aggregate(s)). The monoclonal antibodies may be administered, in therapeutic amounts, to human or animal subjects to reduce the toxicity of the prefibrillar aggregate, thereby preventing or limiting the formation of amyloid deposits and the associated occurrence or progression of a disease or disorder in which amyloid deposits form within the brain or nervous tissue. Examples of such amyloid diseases include, but are not necessarily limited to, Alzheimer's Disease, early onset Alzheimer's Disease associated with Down's syndrome, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, sheep scrapie, and mink spongiform encephalopathy, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, Creutzfeld Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru, fatal familial insomnia, chronic wasting syndrome, familial amyloid polyneuropathy, frontotemporal dementia, type II diabetes, systemic amyloidosis, serum amyloidosis, British familial dementia, Danish familial dementia, macular degeneration and cerebrovascular amyloidosis. The monoclonal antibodies of the present invention are identified as follows: 354B85.1 (clone #56), 354B85.1 (clone #38), 354B85.1 (clone #45), 354B133, 354B256, and 354B273. These clones were prepared by immunizing mice with a conformationally-constrained antigen consisting of amyloid Aβ covalently coupled to colloidal gold via a thioester linkage.

[0011] In accordance with the invention, the prefibrillar aggregate may have a molecular weight in a range of about 1 kDa to about 100,000,000 kDa. Also, the prefibrillar aggregate may comprise any suitable number of monomers. For example, In some specific embodiments the prefibrillar aggregate may comprise five monomers and in other embodiments, the prefibrillar aggregate may comprise eight monomers.

[0012] Still further in accordance with the invention, the amyloid peptide monomers and/or amyloid fibrils may be substantially free of the conformational epitope to which the monoclonal antibody binds.

[0013] Still further in accordance with the invention, the monoclonal antibodies my be coupled to colloidal gold or may be administered concomitantly with gold or gold containing preparations to inhibit certain

[0014] Still further aspects and objects of the present invention may be understood from the detailed description and examples set forth herebelow.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] FIG. 1 is a table comparing the effects of several monoclonal antibodies of the present invention.

[0016] FIG. 2 shows dot blot data obtained for several monoclonal antibodies of the present invention.

DETAILED DESCRIPTION

DEFINITIONS

[0017] As used in this patent application and/or in PCT International Application PCT/US2003/028829 (Publication No. WO 2004/024090 A2) which is incorporated by reference, the following terms shall have the following meanings:

[0018] The term "adjuvant" refers to a compound that when administered in conjunction with an antigen augments the immune response to the antigen, but when administered alone does not generate an immune response to the antigen. Adjuvants can augment an immune response by several mechanisms including lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages.

[0019] The term "A_{β}" peptide" refers to peptides which comprise low molecular weight soluble oligomers, prefibrillar aggregates, fibrils and amyloid deposits each associated with AD. Amyloid A_{β} peptides include, without limitation, A_{β} 39, A_{β} 40, A_{β} 41 A_{β} 42 and A_{β} 43 which are 39, 40, 41, 42 and 43 amino acid amino acids in length, respectively.

[0020] An "amyloid peptide" is a peptide that is present in amyloid forms including amyloid peptide intermediates, low molecular weight soluble oligomers, amyloid fibrils and amyloid plaques.

[0021] The term "antibody" is used to include intact antibodies and binding fragments thereof, including but not limited to, for example, full-length antibodies (e.g., an IgG antibody) or only an antigen binding portion (e.g.,a Fab, F(ab')$_2$ or scFv fragment). Typically, fragments compete with the intact antibody from which they were derived for specific binding to an antigen. Optionally, antibodies or binding fragments thereof, can be chemically conjugated to, or expressed as, fusion proteins with other proteins. "Anti-oligomer antibody" or "Anti-oligomer" refer to an antibody that binds to amyloid peptide aggregate intermediates but does not bind to or does not specifically bind to amyloid peptide monomers, dimers, trimers or tetramers.

[0022] Compositions or methods "comprising" one or more recited elements may include other elements not specifically recited. For example, a composition that comprises an amyloid A_{β} peptide may encompass both an isolated amyloid A peptide as a component of a larger polypeptide sequence or as part of a composition which includes multiple elements.

[0023] The term "epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond or a site on a molecule against which an antibody will be produced and/or to which an antibody will bind. For example, an epitope can be recognized by an antibody defining the epitope.

[0024] A "linear epitope" is an epitope wherein an amino acid primary sequence comprises the epitope recognized. A

linear epitope typically includes at least 3, and more usually, at least 5, for example, about 8 to about 10 amino acids in a unique sequence.

[0025] A "conformational epitope", in contrast to a linear epitope, is an epitope wherein the primary sequence of the amino acids comprising the epitope is not the sole defining component of the epitope recognized (e.g., an epitope wherein the primary sequence of amino acids is not necessarily recognized by the antibody defining the epitope). Typically a conformational epitope comprises an increased number of amino acids relative to a linear epitope. With regard to recognition of conformational epitopes, the antibody recognizes a 3-dimensional structure of the peptide or protein. For example, when a protein molecule folds to form a three dimensional structure, certain amino acids and/or the polypeptide backbone forming the conformational epitope become juxtaposed enabling the antibody to recognize the epitope. Methods of determining conformation of epitopes include but are not limited to, for example, x-ray crystallography 2-dimensional nuclear magnetic resonance spectroscopy and site-directed spin labeling and electron paramagnetic resonance spectroscopy. See, for example, Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed. (1996), the disclosure of which is incorporated in its entirety herein by reference.

[0026] The term "immunological response" or "immune response" relates to the development of a beneficial humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against an amyloid peptide in a recipient patient. Such a response can be an active response induced by administration of monoclonal antibody or a passive response induced by administration of antibody or primed T-cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in association with Class I or Class II MHC molecules to activate antigen-specific CD4$^+$ T helper cells and/or CD8$^+$ cytotoxic T cells. The response may also involve activation of monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity.

[0027] An "monoclonal antibodyic agent" or "monoclonal antibody" or "antigen" is capable of inducing an immunological response against itself upon administration to asubject, optionally in conjunction with an adjuvant.

[0028] "Isolated" means purified, substantially purified or partially purified. Isolated can also mean present in an environment other than a naturally occurring environment. For example, an antibody that is not present in the whole blood serum in which the antibody would ordinarily be found when naturally occurring is an isolated antibody.

[0029] "Low molecular weight aggregate", "low molecular weight amyloid aggregate", "low molecular weight oligomer" and "low molecular weight soluble oligomer" refer to amyloid peptides present in aggregates of less than four or five peptides. In one specific example, low molecular weight A_{β} refers to the low molecular weight soluble oligomers found associated with AD.

[0030] The term "patient" includes human and other animal subjects that receive therapeutic, preventative or diagnostic treatment or a human or animal having a disease or being predisposed to a disease.

[0031] "Prefibrillar aggregates", "micellar aggregates", "high molecular weight aggregation intermediates,""high molecular weight amyloid peptide aggregates", "high molecular weight soluble amyloid peptide aggregates""a-myloid peptide aggregates", "soluble aggregate intermediates", "amyloid oligomeric intermediates", "oligomeric intermediates" and "oligomeric aggregates" or simply, "intermediates" refer to aggregations which include more than three individual peptide or protein monomers, for example, more than four peptide or protein monomers. The upper size of prefibrillar aggregates includes aggregations of oligomers which form spherical structures or micelles and stings of micelles which lead to fibril formation.

[0032] "Annular protofibrils" are a particular subset of prefibrillar aggregates in which 3 to 10 spherical oligomer subunits are arranged in an annular or circular fashion with a hollow center that appears as a pore in electron or atomic force micrographs.

[0033] The molecular weight of a prefibrillar aggregate may be in a range of about 10 kDa to about 100,000,000 KDa, for example, about 10 kDa to about 10,000,000 or 1,000,000 KDa. However, this size range is not intended to be limiting and prefibrillar aggregates are not defined by a molecular weight range.

[0034] "Protofibrils" are prefibrillar aggregates which include spherical structures comprising amyloid A_{β} peptides that appear to represent strings of the spherical structures forming curvilinear structures.

[0035] "Specific binding" between two entities means an affinity of at least 10^6, 10^7, 10^8 10^9 M^{-1}, or 10^{10} M^{-1}. Affinities greater than 10^8 M^{-1} are preferred for specific binding.

[0036] The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 65 percent sequence identity, for example, at least 80 percent or 90 percent sequence identity, or at least 95 percent sequence identity or more, for example, 99 percent sequence identity or higher.

[0037] Preferably, residue positions in an alignment which are not identical differ by conservative amino acid substitutions, i.e., substitution of an amino acid for another amino acid of the same class or group. Some amino acids may be grouped as follows: Group I (hydrophobic side chains): leu, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Non-conservative substitutions may include exchanging a member of one of these classes for a member of another class.

[0038] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm may then be used to calculate the percent sequence identity for the test sequence (s) relative to the reference sequence, based on the designated program parameters. Optimal alignment of sequences for

comparison can be conducted, for example, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48: 443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection.

[0039] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215: 403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Typically, default program parameters can be used to perform the sequence comparison, although customized parameters can also be used. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix, see for example, Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89,10915 (1989). Conservative substitutions involve substitutions between amino acids in the same class.

[0040] A "therapeutic agent" or "therapeutic" is a substance useful for the treatment or prevention of a disease in a patient. Therapeutic agents of the invention are typically substantially pure. This means that an agent is typically at least about 50% w/w (weight/weight) pure, as well as being substantially free from proteins and contaminants which interfere with the efficacy of the therapeutic. The agents may be at least about 80% w/w and, more preferably at least 90 % w/w or about 95% w/w in purity. However, using conventional protein purification techniques, homogeneous peptides of 99% w/w or more can be produced.

EMBODIMENTS AND EXAMPLES

[0041] Amyloid diseases are characterized by the accumulation of amyloid plaques or precursors to amyloid plaques in patients or the predisposition to the accumulation of amyloid plaques or precursors to amyloid plaques in patients. One of the primary constituents of amyloid plaques are amyloid peptides. The general conformation of amyloid peptides may vary from disease to disease, but often the peptide has a characteristic-pleated sheet structure. Amyloid peptides include peptides and proteins of about 10 or about 20 amino acids to about 200 amino acids in length. Though this size range is not intended as a limitation and amyloid peptides or proteins having fewer or more amino acids are contemplated in the present invention.

[0042] Prefibrillar aggregates are intermediates in the production of insoluble fibrils that accumulate in amyloid plaques of humans or animals having a disease characterized by amyloid deposits, for example, Alzheimer's disease. Prefibrillar aggregates include aggregates which may be as small as four amyloid peptides, as small as five amyloid peptides, as small as six amyloid peptides, as small as seven amyloid peptides or as small as eight amyloid peptides. In one embodiment, prefibrillar aggregates are micellar aggregates or micelles or strings of micelles. Prefibrillar aggregates are effective to form a conformational epitope which is recognized by an antibody of the present invention.

[0043] The conformational epitopes found on prefibrillar aggregates are substantially not found in the native precursor proteins for amyloid peptides, for example, amyloid peptide monomers, dimers, trimers or tetramers nor in the mature amyloid fibers that are defined by their characteristic cross x-ray fiber diffraction pattern or in amyloid plaques. The prefibrillar aggregates that contain the specific polypeptide structure which results in conformational epitopes that are recognized by antibodies of the present invention have a size range of approximately a pentamer, a hexamer, a heptamer or an octamer to micellar forms or protofibrils which have a molecular weight in excess of 1,000,000 Daltons. Antibodies of the invention are effective to bind to these epitopes.

[0044] Monoclonal antibodies of the present invention are specific for a conformation-dependent epitope associated with amyloid oligomers or protofibrils. The monoclonal antibodies may be prepared by immunizing mice with a conformationally-constrained antigen consisting of amyloid Aβ covalently coupled to colloidal gold via a thioester linkage. FIG. 1 shows in diagrammatic form an example of how such monoclonal antibodies may be produced. Such monoclonal antibodies will provide for diagnostic and therapeutic uses. The antibody is also useful for determining the three dimensional structure of amyloid oligomers bound to the antibody by co-crystallization of the antibody Fab with the antigen and X-ray crystallography.

[0045] Supernatants from hybridoma fusions were screned by ELISA by Strategic Biosolutions and the same supernatants were sent to UCI for further analysis by Dr. Rakez Kayed, Monica Siegenthaler and Maya Hatch by dot blot assay. For ELISA assay, 100 ng of soluble oligomeric or fibrillar Aβ42 was suspended in plating buffer and used to coat hyBond ELISA plates for 1 hr to overnight. After coating the wells were blocked with 300 ul 10 BSA in Tris-buffered saline, 0.01% Tween 40 (TBST) at 37 degrees C. for 1 hr. Tissue culture supernatant from the hybridomas was added to the wells at 1:200, 1:500, 1:1000, 1:2000 and 1:5000 and incubated at 37 degrees for 1 hr. The plates were washed 3× with phosphate buffered saline (PBS) and 100 ul of goat anti mouse-horseradish peroxidase conjugate 1:10, 000 dilution was added to each well and incubated for 1 hr. the plates were washed 3 times with PBS and then assayed for HRP activity by adding 100 ul of color diction substrate, TMB. The plates were read at 450 nm. Clones that show high reactivity against oligomers and low reactivity against monomer and fibrils were selected.

[0046] Dot Blot Assay:

[0047] Monomer, oligomer and fibrillar samples of Aβ42 (100 ng) were applied to a nitrocellulose membrane, dried and blocked with 10% BSA in TBST. Tissue culture supernatant from the hybridomas was added to each strip at 1:200, 1:500, 1:1000, 1:2000 and 1:5000 and Incubated at 37 degrees for 1 hr. The strips were washed 3 times with PBS, and incubated at 37 degrees for 1 hr with goat anti mouse-horseradish peroxidase conjugate 1:10,000. The strips were washed 3 times with PBS and the antibody binding visualized by enhanced chemiluminescence (ECL). A typical dot blot is shown in FIG. 2 for clones 354B85.1 clone #38, and 354B85.1 clone #45, 354B256, and 354B273. Lane 1 is Aβ42 monomer. Lane 2 is Aβ42 oligomers. Lane 3 is Aβ42 fibrils. Lane 4 is human lysozyme oligomers.

[0048] FIG. 1 contains a summary of results pertaining to the screening of antibodies that are specific for a conforma-

tional epitope that is common to amyloid oligomeric intermediates. It summarizes the results of fusion 31 B from mouse 1867/11 #5684 that was vaccinated with a micelle molecular mimic consisting of a conformationally constrained Aβ40 thioester coupled to colloidal gold. The mouse was boosted with soluble Aβ40 oligomers 3 days before the spleen was removed and used for hybridoma production in order to increase the number of circulating B cells to useable levels. The first column lists the hybridoma clone label. The second column lists the results of ELISA assay using ELISA plates containing rows of soluble low MW Aβ (sol), oligomeric intermediates (interm) and amyloid fibrils (fibril). The numbers are optical absorbance values in absorbance units and represent the extent to which the different clones recognize the different conformations of the Aβ adsorbed to the plate. A low or background number in the sol and fibril column indicates a lack of binding or recognition, while a high value in the interm column indicates a high degree of recognition or binding. Clones with a low number for sol and fibrils with a high number for interm indicate a high degree of specificity for the soluble oligomer conformation dependent epitope. Examples of clones exhibiting a high degree of specificity for soluble oligomers and not low MW soluble Aβ or fibrils include, but are not limited to clones 354B85.1 (clone #56), 354B85.1 (clone #38), 354B85.1 (done #45), 354B256 and 354B273

[0049] Each of the following amyloid peptides have been shown to form amyloid peptide aggregates which produce a conformational epitope recognized by the antibodies of the present invention, for example, antibodies produced against A peptide oligomeric intermediates. Some of these peptides are present in amyloid deposits of humans or animals having a disease characterized by the amyloid deposits. The present invention is not limited to the listed peptide or protein sequences or the specific diseases associated with some of the sequences. The present invention contemplates antibodies as described herein binding to other amyloid peptide aggregates or all other amyloid peptide aggregates. In particular, the present invention contemplates and includes the application of methods and compositions of the present invention to other peptide or protein sequences which form amyloid precursor aggregates associated with other diseases.

A40 (SEQ ID NO 1)
DAEFRHDSGYEVHHQKLVFF AEDVGSNKGA IIGLMVGGVV

A42 (SEQ ID NO 2)
DAEFRHDSGY EVHHQKLVFF AEDVGSNKGA IIGLMVGGVV IA

Human IAPP (SEQ ID NO 3)
KCNTATCATQ RLANFLVHSS NNFGAILSST NVGSNTY

Human Prion 106–126 (SEQ ID NO 4)
KTNMKHMAGA AAGAVVGGLG

[0050] Stefani and coworkers (Bucciantini et al (2002) Nature 416, 507-511) have recently reported that amyloid peptide aggregates formed from non-disease-related proteins are inherently cytoxic, suggesting that they may have a structure in common with disease related amyloid peptides. Non-disease related amyloid peptide aggregates comprising the following non-disease related amyloid peptides are also shown to bind to the antibodies of the present invention.

Poly glutamine synthetic peptide KK(Q40)KK
(SEQ ID NO 5)
KKQQQQQQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQQQQ
QQKK

Human Lysozyme (SEQ ID NO 6)
MKALIVLGLV LLSVTVQGKV FERCELARTL KRLGMDGYRG
SLANWMCLA KWESGYNTRA TNYNAGDRST DYGIFQINSR
YWCNDGKTPG AVNACHLSCS ALLQDNIADA VACAKRVVRD
PQGIRAWVAW RNRCQNRDVR QYVQGCGV

Human Insulin (SEQ ID NO 7)
MALWMRLLPL LALLALWGPD PAAAFVNQHL CGSHLVEALY
LVCGERGFFY TPKTRREAED LQVGQVELGG GPGAGSLQPL
ALEGSLQKRG IVEQCCTSIC SLYQLENYCN

Human Transthyretin (SEQ ID NO 8)
MASHRLLLLC LAGLVFVSEA GPTGTGESKC PLMVKVLDAV
RGSPAINVAV HVFRKAADDT WEPFASGKTS ESGELHGLTT
EEEFVEGIYK VEIDTKSYWK ALGISPFHEH AEVVFTANDS
GPRRYTIAAL LSPYSYSTTA VVTNPKE

Human Alpha Synuclein (SEQ ID NO 9)
MDVFMKGLSK AKGVVAAAE KTKQGVAEAA GKTKEGVLYV
GSKTKEGVVH GVATVAEKTK EQVTNVGGAV VTGVTAVAQK
TVEGAGSIAA ATGFVKKDQL GKNEEGAPQE GILEDMPVDP
DNEAYEMPSE EGYQDYEPEA

[0051] In addition, oligomeric intermediates formed from variants and fragments of wild type A42, A40 including, without limitation A42 (A21G) Flemish mutation), A42 (E22Q) Dutch mutation, A42 (E22G) Arctic mutation, A42 (D23N) Iowa mutation, A40 (A21G) Flemish mutation), A40 (E22Q) Dutch mutation, A40 (E22G) Arctic mutation, A40 (D23N) Iowa mutation, A40 (E22Q &D23N) Dutch & Iowa mutations, A 3-42 (pGlu 3), A 3-40 (pGlu 3), A8-42, A17-42, A1-16, A3-11, A25-35, A4-16 (3 analogues, Cys^{16} A4-16, 16, and Ala^{10} A4-16), His6 A40C40 (6 histidines appended to the amino terminus of AβC40) are recognized by the antibodies of the present invention. Other oligomeric intermediates recognized by antibodies of the invention include, without limitation, oligomeric intermediates formed from IAPP(C2AandC7A) where alanine is substituted for the naturally occurring cysteine in IAPP, Polyglutamine KKQ40KK or poly glutamine where the number of Q residues is greater than 32, Calcitonin, TTR and its mutants TTR Pro^{55}, TTR Phe^{78}, vitronictin, poly Lysine, poly arginine, serum amyloid A, cystantin C, IgG kappa light chain, oligomeric intermediates produced from other amyloid peptides disclosed herein and amyloid intermediates associated with amyloid diseases disclosed herein.

[0052] The present invention provides for amyloid disease therapeutics which induce a specific immune response against amyloid oligomeric intermediates. Therapeutics of the invention include antibodies that specifically bind to oligomeric intermediates. Such antibodies can be monoclonal as described in this application or polyclonal as described in PCT International Application No. PCT/US2003/028829, which is incorporated herein by reference. In one useful embodiment, the antibodies bind to a conformational epitope. The production of non-human monoclonal antibodies of the present invention (e.g., murine or rat) can be accomplished by, for example, immunizing the animal with an oligomeric intermediate mimic of the invention. Also contemplated is immunizing the animal with a purified amyloid intermediate.

[0053] Humanized forms of mouse antibodies of the invention can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. See Queen et al., Proc. Natl. Acad. Sci. USA 86,10029-10033 (1989) and WO 90/07861 (incorporated by reference for all purposes).

[0054] Human antibodies may be obtained using phage-display methods. See, for example, Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047. In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Phage displaying antibodies with a desired specificity are selected by affinity enrichment. Human antibodies against oligomeric intermediates may also be produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus and an inactivated endogenous immunoglobulin locus. See, for example, Lonberg et al., W093/12227 (1993); Kucherlapati, WO 91/10741 (1991) (each of which is incorporated by reference in its entirety for all purposes). Human antibodies can be selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody. Such antibodies are particularly likely to share the useful functional properties of the mouse antibodies.

[0055] Human or humanized antibodies can be designed to have IgG, IgD, IgA and IgE constant region, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Antibodies can be expressed as tetramers containing two light and two heavy chains, as separate heavy chains, light chains, as Fab, Fab' $F(ab')_2$ and Fv, or as single chain antibodies in which heavy and light chain variable domains are linked through a spacer.

[0056] In certain instances it may be desirable to combine one or more monoclonal anibodies of the present invention with a suitable carrier. Suitable carriers include serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria, such as diphtheria, *E. coli*, cholera, or *H. pylori*, or an attenuated toxin derivative. Other carriers which may act as adjuvants for stimulating or enhancing an immune response include cytokines such as IL-1, IL-1 and peptides, IL-2, INF, IL-10, GM-CSF, and chemokines, such as MIP1 and and RANTES.

[0057] Human or animal subjects or patients amenable to treatment with monoclonal antibodies of the present invention include individuals at risk of amyloid disease but not showing symptoms, as well as those who already show symptoms or other evidence of amyloid disease. In the case of certain amyloid diseases including AD, virtually anyone is at risk of suffering from the disease.

[0058] Therefore, monoclonal antibodies of the present invention could be administered prophylactically, for example, as a vaccine, to the general population without any assessment of the risk of the subject patient. The present methods are especially useful for individuals who do have a known genetic risk of an amyloid disease, for example, AD. Such individuals may include those having relatives who have experienced an amyloid disease, and those whose risk is determined by analysis of genetic or biochemical markers or who exhibit symptoms or prodromes indicative of the potential for development of, or the actual presence of, such diseases. For example, genetic markers of risk toward AD include mutations in the APP gene, particularly mutations at position 717 and positions 670 and 671 referred to as the Hardy and Swedish mutations respectively (see Hardy, TINS, supra). Other markers of risk for AD are mutations in the presenilin genes, PSI and PS2, and ApoE4, family history of AD, hypercholesterolemia or atherosclerosis.

[0059] Symptoms of amyloid disease are apparent to a physician of ordinary skill. For example, Individuals presently suffering from Alzheimer's disease can be recognized from characteristic dementia, as well as the presence of risk factors described above. In addition, a number of diagnostic tests are available for identifying individuals who have amyloid diseases. For example, in the case of AD these include measurement of CSF tau and $A_{\beta}42$ levels. Elevated tau and decreased $A_{\beta}42$ levels signify the presence of AD.

[0060] In asymptomatic patients, treatment can begin at any age, for example, at the age of 10, 20, 30, 40, 50, 60 or 70. Treatment may entail one or more doses, for example, multiple dosages over a period of time. Treatment can be monitored by assaying antibody, or activated T-cell or B-cell responses to the therapeutic (for example, oligomeric intermediate mimic) or assaying the levels of prefibrillar aggregate present, each over time. In one embodiment, treatment by administering a single therapeutic of the invention, such as a preparation containing a single monoclonal antibody of the invention, may serve as a treatment for or preventive measure against more than one amyloid disease, for example all amyloid diseases.

[0061] In prophylactic applications, compositions of the invention or medians are administered to a patient susceptible to, or otherwise at risk of, a particular disease in an amount sufficient to eliminate or reduce the risk or delay the outset of the disease. In therapeutic applications, compositions or medians are administered to a patient suspected of, or already suffering from such a disease in an amount sufficient to cure, or at least partially arrest, the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a therapeutically-or pharmaceutically-effective dose. In both prophylactic and therapeutic regimes, therapeutics are usually administered in several dosages until a sufficient immune response has been achieved. Typically, the immune response is monitored and repeated dosages are given if the immune response starts to fade.

[0062] Effective doses of the compositions of the present invention, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human, but in some diseases, such as mad cow disease, the patient can be a nonhuman mammal, such as a bovine or in the case of Alzheimer's disease, the patient may be a dog. Treatment dosages need to be titrated to optimize safety and efficacy. For passive immunization with an antibody, the dosage ranges from about 0.0001 mg/kg of body weight to about 100 mg/kg of body weight, and more usually about 0.01 mg/kg of body weight to about 5 mg/kg of body weight of the host. The amount of monoclonal antibody to be administered may depend on whether any adjuvant is also administered, with higher dosages being required in the absence of

adjuvant. For example, 0.1 to 100 cc of a solution containing approximately 1% by weight of the desired monoclonal antibody(ies) my be injected subcutaneously, thereby delivering a dose of 1 mg to 1 g of the monoclonal antibody(ies) per injection. The timing of injections can vary significantly from once a day, to once a year, to once a decade. One typical regimen consists of an immunization followed by booster injections at 6 weekly intervals. Another regimen consists of an immunization followed by booster injections 1,2 and 12 months later. Another regimen entails an injection every two months for life. Alternatively, booster injections can be on an irregular basis as indicated by monitoring of immune response.

[0063] Therapeutics for inducing an immune response can be administered by any suitable route of administration, for example, parenteral, topical, intravenous, oral, subcutaneous, intraperitoneal, intranasal or intramuscular. The most typical route of administration is subcutaneous although others can be equally effective. The next most common is intramuscular injection. This type of injection is most typically performed in the arm or leg muscles. Intravenous injections as well as intraperitoneal injections, intraarterial, intracranial, or intradermal injections may also be effective in generating an immune response. In some methods, therapeutics are injected directly into a particular tissue where deposits have accumulated or may accumulate.

[0064] Monoclonal antibodies of the invention can optionally be administered in combination with other agents that are at least partly effective in treatment of amyloidogenic disease. In the case of Alzheimer's and Down's syndrome, in which amyloid deposits occur in the brain, therapeutics of the invention can also be administered in conjunction with other agents that increase passage of the compositions of the invention across the blood-brain barrier. For example, as described in detail herebelow, anti-inflammatory dosages of colloidal gold or gold salts may be administered concomitantly (e.g., before, concurrently with or after) the monoclonal antibody to deter the brain inflammation associated with AD and other amyloid diseases.

[0065] Monoclonal antibodies of the invention may sometimes be administered in combination with an adjuvant. A variety of adjuvants can be used in combination with an monoclonal antibody of the invention to elicit an immune response. Preferred adjuvants augment the intrinsic response to an monoclonal antibody without causing conformational changes in the monoclonal antibody that affect the qualitative form of the response. Preferred adjuvants include alum, 3 de-O-acylated monophosphoryl lipid A (MPL) (see GB 2220211). QS21 is a triterpene glycoside or saponin isolated from the bark of the Quillaja Saponaria Molina tree found in South America (see Kensil et al., in Vaccine Design: The subunit and Ajuvant Approach (eds. Powell & Newman, Plenum Press, NY, 1995); and U.S. Pat. No. 5,057,540). Other adjuvants are oil in water emulsions, such as squalene or peanut oil, optionally in combination with immune stimulants, such as monophosphoryl lipid A. See, for example, Stoute et al., N. Engl. J. Med. (1997) 336,86-91. Another useful adjuvant is CpG described in Bioworld Today, Nov. 15, 1998. Alternatively, a monoclonal antibody can be coupled to an adjuvant. However, such coupling should not substantially change monoclonal antibody so as to affect the nature of the immune response thereto. Adjuvants can be administered as a component of a therapeutic composition with an active agent or can be administered separately, before, concurrently with, or after administration of the therapeutic.

[0066] A preferred class of adjuvants is aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate. Such adjuvants can be used with or without other specific immunostimulating agents such as MPL or 3-DMP, QS21, polymeric or monomeric amino acids such as polyglutamic acid or polylysine.

[0067] Another class of adjuvants is oil-in-water emulsion formulations. Such adjuvants can be used with or without other specific immunostimulating agents such as muramyl peptides (for example, N-acetylmuramyl-L-threonyl-D-isoglutamine (thr-MDP), -acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanine-2-(1'-2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), N-acetylglucsaminyl-N-acetylmuramyl-L-Al-D-isoglu-L-Ala-dipalmitoxy propylamide (DTP-nPP) theramide™, or other bacterial cell wall components. Oil-in-water emulsions include (a) MF59 (WO 90/14837), containing 5% Squalene, 0.5% Tween 80 and 0.5% Span 85 (optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton Mass.), (b) SAF, containing 10% Squalane, 0.4% Tween 80,5% pluronic-blocked polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, Mont.) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL+CWS (Detox™)

[0068] Another class of preferred adjuvants is saponin adjuvants, such as Stimulons (QS21, Aquila, Worcester, Mass.) or particles generated therefrom such as ISCOMs (immunostimulating complexes) and ISCOMATRIX. Other adjuvants include Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA). Other adjuvants include cytokines, such as interleukins, for example, IL-1, IL-2, and IL-12, macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF) and/or chemokines such as CXCL10 and CCL5.

[0069] An adjuvant can be administered with an monoclonal antibody as a single composition, or can be administered before, concurrent with or after administration of the monoclonal antibody. Monoclonal antibody and adjuvant can be packaged and supplied in the same vial or can be packaged in separate vials and mixed before use. Monoclonal antibody and adjuvant are typically packaged with a label indicating the intended therapeutic application. If monoclonal antibody and adjuvant are packaged separately, the packaging typically includes instructions for mixing before use. The choice of an adjuvant and/or carrier depends on the stability of the vaccine containing the adjuvant, the route of administration, the dosing schedule, the efficacy of the adjuvant for the species being vaccinated, and, in humans, a pharmaceutically acceptable adjuvant is one that has been approved or is approvable for human administration by pertinent regulatory bodies. For example, Complete Freund's adjuvant is not suitable for human administration.

Optionally, two or more different adjuvants can be used simultaneously. Preferred combinations include alum with MPL, alum with QS21, MPL with QS21, and alum, QS21 and MPL together. Also, Incomplete Freund's adjuvant can be used (Chang et al., Advanced Drug Delivery Reviews 32,173-186 (1998)), optionally in combination with any of alum, QS21, and MPL and all combinations thereof.

[0070] Compositions of the invention are often administered as pharmaceutical compositions comprising a variety of other pharmaceutically acceptable components. See Remington's Pharmaceutical Science (15th ed., Mack Publishing Company, Easton, Pa., 1980). The preferred form depends on the intended mode of administration and therapeutic application. The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonmonoclonal antibodyic stabilizers and the like. However, some reagents suitable for administration to animals, such as complete Freund's adjuvant are not typically included in compositions for human use.

[0071] Pharmaceutical compositions can also include large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids and copolymers (such as latex functionalized sepharose, agarose, cellulose, and the like), polymeric amino acids, amino acid copolymers, and lipid aggregates (such as oil droplets or liposomes). Additionally, these carriers can function as immunostimulating agents (i. e., adjuvants).

[0072] For parenteral administration, compositions of the invention can be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid such as water oils, saline, glycerol, or ethanol.

[0073] Auxiliary substances, such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in compositions. Other components of pharmaceutical compositions are those of petrolcum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

[0074] Compositions may be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The preparation also can be emulsified or encapsulated in liposomes or micro particles such as polylactide, polyglycolide, or copolymer for enhanced adjuvant effect, as discussed above. See Langer, Science (1990) 249, 1527and Hanes, Advanced Drug Delivery Reviews (1997) 28,97-119. The compositions of this invention can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained or pulsatile release of the active ingredient.

[0075] Additional formulations suitable for other modes of administration include oral, intranasal, and pulmonary formulations, suppositories, and transdermal applications.

[0076] For suppositories, binders and carriers include, for example, polyalkylene glycols or triglycerides; such suppositories can be formed from mixtures containing the active ingredient in the range of 0.5% to about 10%, for example, about 1% to about 2%. Oral formulations include excipients, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and may contain about 10% about 95% of active ingredient, for example, about 25% to about 70%.

[0077] Topical application can result in transdermal or intradermal delivery. Topical administration can be facilitated by co-administration of the composition with cholera toxin or detoxified derivatives or subunits thereof or other similar bacterial toxins. See Glenn et al., Nature (1998) 391,851. Co-administration can be achieved by using the components as a mixture or as linked molecules obtained by chemical crosslinking or expression as a fusion protein.

[0078] Alternatively, transdermal delivery can be achieved using a skin path or using transferosomes. See for example, Paul et al., Eur. J. Immunol. (1995) 25,3521-24; Cevc et al., Biochem. Biophys. Acta (1998) 1368,201-15.

Concomitant Administration of Gold or Other Antiinflammatory

[0079] The anti-inflammatory effects of gold are well established. For example, injectable colloidal gold preparations (Myochrysine™ or Solganal™) are commercially available for the treatment of rheumatoid arthritis. A gold preparation for oral administration (Auranofin™) is also available. Inflammation of in the brain is thought to be a cause or contributing factor Alzheimer's Disease, primarily because amyloid-beta (protein) which is found in the brains of Alzheimer's patients is known to be an inflammatory protein. In view of this, others have proposed the use of non-steroidal anti-inflammatory drugs such as rofecoxib (Vioxx) and naproxen (Aleve) to slow the progression of Alzheimer's Disease.

[0080] Applicants have determined, on the basis of histopathological observations, that the subcutaneous administration of colloidal gold can reduce microglial activation in the brains of mice modeling for amyloid disease. The present invention includes the administration of colloidal gold, gold salts or other antiinflammatory agents to the subject in an amount that is therapeutically effective to decrease neural inflammation. In some cases, the gold or anti-inflammatory agent may be combined with the monoclonal antibody. In other cases, the gold or anti-inflammatory agent may be administered separately from the monoclonal antibody. Any syitable dose, dosing schedule or route of administration may be used. For example, commercially available gold preparations for treatment of rheumatoid arthritis may be administered by the same routes of administration (subcutaneous injection of Myochrysine™ or solganaI™ or oral administration of AuranofiN™ and dosages/dosing schedules recommended for treatment of rheumatoid arthritis.

[0081] Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practised within the scope of the appended claims. All publications and patent documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

What is claimed is:

1. A composition comprising an isolated monoclonal antibody which binds to a conformational epitope of a prefibrillar aggregate which forms in a human or animal contributing to amyloid fibril formation, said monoclonal antibody being specific for a conformation-dependent epitope that is preferentially displayed by oligomeric conformations of Aβ and other amyloids.

2. A composition according to claim 1 wherein the monoclonal antibody is effective to reduce the toxicity of the prefibrillar aggregate.

3. A composition according to claim 1 wherein the prefibrillar aggregate has a molecular weight in a range of about 1 kDa to about 100,000,000 kDa.

4. A composition according to claim 1 wherein the prefibrillar aggregate comprises five monomers.

5. A composition according to claim 1 wherein the prefibrillar aggregate comprises eight monomers.

6. A composition according to claim 1 wherein amyloid peptide monomers are substantially free of the conformational epitope.

7. A composition according to claim 1 wherein amyloid fibrils are substantially free of the epitope.

8. A composition according to claim 1 wherein the prefibrillar aggregate comprises a toxic species.

9. A composition according to claim 1 wherein the prefibrillar aggregate is present in a human or animal having a disease characterized by amyloid deposits.

10. A composition according to claim 9 wherein the disease is selected from the group consisting of Alzheimer's Disease, early onset Alzheimer's Disease associated with Down's syndrome, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, sheep scrapie, and mink spongiform encephalopathy, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, Creutzfeld Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru, fatal familial insomnia, chronic wasting syndrome, familial amyloid polyneuropathy, frontotemporal dementia, type II diabetes, systemic amyloidosis, serum amyloidosis, British familial dementia, Danish familial dementia, macular degeneration and cerebrovascular amyloidosis.

11. A composition according to claim 9 wherein the disease is Alzheimer's.

12. A composition according to claim 1 wherein the composition is a pharmaceutical composition.

13. A preparation comprising at least one monoclonal antibody according to claim 1 in combination with at least one anti-inflammatory agent.

14. A preparation according to claim 13 wherein the anti-inflammatory agent comprises gold.

15. A composition comprising a monoclonal antibody which binds to an epitope of a prefibrillar aggregate which forms in a human or animal contributing to an amyloid fibril formation wherein the amyloid fibril is substantially free of the epitope.

16. A composition according to claim in 15 wherein the prefibrillar aggregate comprises a toxic species.

17. A composition according to claim 15 wherein amyloid peptide monomers are substantially free of the epitope.

18. A composition according to claim 15 wherein the monoclonal antibody is effective to reduce the toxicity of the prefibrillar aggregate.

19. A composition according to claim 15 wherein the prefibrillar aggregate has a molecular weight in a range of about 1 kDa to about 100,000,000 kDa.

20. A composition according to claim 15 wherein the prefibrillar aggregate comprises five monomers.

21. A composition according to claim 15 wherein the prefibrillar aggregate comprises eight monomers.

22. A composition according to claim 15 wherein the prefibrillar aggregate is present in a human or animal having a disease characterized by amyloid deposits.

23. A composition according to claim 22 wherein the disease is selected from the group consisting of Alzheimer's, early onset Alzheimer's associated with Down's syndrome, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, sheep scrapie, and mink spongiform encephalopathy, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, Creutzfeld Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru, fatal familial insomnia, chronic wasting syndrome, familial amyloid polyneuropathy, frontotemporal dementia, type II diabetes, systemic amyloidosis, serum amyloidosis, British familial dementia, Danish familial dementia, macular degeneration and cerebrovascular amyloidosis.

24. A composition according to claim 22 wherein the disease is Alzheimer's Disease.

25. A preparation comprising at least one monoclonal antibody according to claim 15 in combination with at least one anti-inflammatory agent.

26. A preparation according to claim 25 wherein the anti-inflammatory agent comprises gold.

27. A composition according to claim 15 wherein the composition is a pharmaceutical composition.

28. A method for treating a disease or condition characterized by amyloid deposits in a human or animal subject, said method comprising the step of:

A. causing a monoclonal antibody to bind to a conformational epitope of a prefibrillar aggregate which forms in a human or animal contributing to fibril formation.

29. A method according to claim 28 wherein step A comprises administering to the subject a therapeutically effective or preventative amount of a monoclonal antibody that has been prepared by immunizing mice with a conformationally-constrained antigen consisting of amyloid Ap covalently coupled to colloidal gold via a thioester linkage.

30. A method according to claim 28 wherein the prefibrillar aggregate comprises a toxic species of prefibrillar aggregate.

31. A method according to claim 30 wherein the monoclonal antibody is effective to reduce toxicity of the prefibrillar aggregate.

32. A method according to claim 28 wherein the prefibrillar aggregate has a molecular weight in a range of about 1 kDa to about 100,000,000 kDa.

33. A method according to claim 28 wherein the prefibrillar aggregate comprises five monomers.

34. A method according to claim 28 wherein the prefibrillar aggregate comprises eight monomers.

35. A method according to claim 28 wherein amyloid peptide monomers are substantially free of the epitope.

36. A method according to claim 28 wherein amyloid fibrils are substantially free of the epitope.

37. A method according to claim 28 wherein the prefibrillar aggregate is present in a human or animal having a disease characterized by amyloid deposits.

38. A method according to claim 28 wherein the disease or condition is selected from the group consisting of Alzheimer's Disease, early onset Alzheimer's Disease associated with Down's syndrome, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, sheep scrapie, and mink spongiform encephalopathy, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, Creutzfeld Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru, fatal familial insomnia, chronic wasting syndrome, familial amyloid polyneuropathy, frontotemporal dementia, type II diabetes, systemic amyloidosis, serum amyloidosis, British familial dementia, Danish familial dementia, macular degeneration and cerebrovascular amyloidosis.

39. A method according to claim 28 wherein the disease is Alzheimer's.

40. A method according to claim 28 wherein the composition is administered by a method selected from the group consisting of intraspinal, intrathecal, oral, transdermal, pulmonary, intravenous, subcutaneous, intranasal, intraarterial, intracranial, intradermal, intraperitoneal, intramuscular, rectal and buccal administration.

41. A method according to claim 28 further comprising the step of:

B. administering to the subject an antiinflamatory agent in an amount that is effective to deter brain inflammation.

42. A method according to claim 41 wherein Step B comprises administering gold or a gold-containing compound to the subject in an amount that is therapeutically effective to decrease neural inflammation.

43. A method according to claim 42 wherein a colloidal gold preparation is administered in Step B.

44. A method according to claim 41 wherein the anti-inflammatory agent is combined with the monoclonal antibody.

45. A method according to claim 41 wherein the anti-inflammatory agent is separate from the monoclonal antibody.

46. A method for treating a disease or condition characterized by amyloid deposits neural tissue in a human or animal subject, said method comprising the step of:

A. causing a monoclonal antibody to bind to an epitope of a prefibrillar aggregate which forms in a human or animal contributing to an amyloid fibril formation wherein the amyloid fibril is substantially free of the epitope.

47. A method according to claim 46 wherein step A comprises administering to the subject a therapeutically effective or preventative amount of a monoclonal antibody such that the monoclonal antibody will bind in accordance with Step A.

48. A method according to claim 46 wherein the monoclonal antibody binds to a conformational epitope of a prefibrillar aggregate that contributes to amyloid fibril formation in the human or animal subject, said monodonal antibody being specific for a conformation-dependent epitope that is preferentially displayed by oligomeric conformations of Aβ and other amyloids.

49. A method according to claim 46 wherein the prefibrillar aggregate has a molecular weight in a range of about 1 kDa to about 100,000,000 kDa.

50. A method according to claim 46 wherein the prefibrillar aggregate comprises five monomers.

51. A method according to claim 46 wherein the prefibrillar aggregate comprises eight monomers.

52. A method according to claim 46 wherein the prefibrillar aggregate comprises a toxic species.

53. A method according to claim 46 wherein the monoclonal antibody is effective to reduce toxicity of the prefibrillar aggregate.

54. A method according to claim 46 wherein amyloid fibrils are substantially free of the epitope.

55. A method according to claim 46 wherein the prefibrillar aggregate comprises a toxic species.

56. A method according to claim 46 wherein the prefibrillar aggregate is present in a human or animal having a disease characterized by amyloid deposits.

57. A method according to claim 46 wherein the disease or condition is selected from the group consisting of Alzheimer's, early onset Alzheimer's associated with Down's syndrome, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, sheep scrapie, and mink spongiform encephalopathy, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, Creutzfeld Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru, fatal familial insomnia, chronic wasting syndrome, familial amyloid polyneuropathy, frontotemporal dementia, type II diabetes, systemic amyloidosis, serum amyloidosis, British familial dementia, Danish familial dementia, macular degeneration and cerebrovascular amyloidosis.

58. A method according to claim 46 wherein the disease or condition is Alzheimer's Disease.

59. A method according to claim 46 wherein the composition is administered by a method selected from the group consisting of intraspinal, intrathecal, oral, transdermal, pulmonary, intravenous, subcutaneous, intranasal, intraarterial, intracranial, intradermal, intraperitoneal, intramuscular, rectal and buccal administration.

60. A method according to claim 46 further comprising the step of:

B. administering to the subject an antiinflamatory agent in an amount that is effective to deter brain inflammation.

61. A method according to claim 60 wherein Step B comprises administering gold or a gold-containing compound to the subject in an amount that is therapeutically effective to decrease neural inflammation.

62. A method according to claim 61 wherein a colloidal gold preparation is administered in Step B.

63. A method according to claim 62 wherein the anti-inflammatory agent is combined with the monoclonal antibody.

64. A method according to claim 63 wherein the anti-inflammatory agent is separate from the monoclonal antibody.

65. A method for making a monoclonal antibody, said method comprising the step of:

A. obtaining a conformational epitope of a prefibrillar aggregate which forms in a human or animal contributing to amyloid fibril formation.

66. The method according to claim 65 wherein step A comprises recovering the monoclonal antibody from a human or animal.

67. A method for making a monoclonal antibody, said method comprising the step of:

A. administering to a human or animal a composition comprising an epitope of a prefibrillar aggregate which forms in a human or animal contributing to an amyloid fibril formation wherein the amyloid fibril is substantially free of the epitope.

68. The method according to claim 67 wherein step A comprises recovering the monoclonal antibody from the human or animal.

69. A method for diagnosing a disease or condition in a human or animal subject, said disease or condition being characterized by the formation of amyloid deposits in neural tissue, said method comprising the step of:

A. combining tissue or fluid from the human or animal subject and a composition comprising or consisting of a monoclonal antibody, said monoclonal antibody being one that binds to a conformational epitope of a prefibrillar aggregate that contributes to amyloid fibril formation.

70. A method according to claim 69 wherein the disease or condition is selected from the group consisting of Alzheimer's, early onset Alzheimer's associated with Down's syndrome, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, sheep scrapie, and mink spongiform encephalopathy, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, Creutzfeld Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru, fatal familial insomnia, chronic wasting syndrome, familial amyloid polyneuropathy, frontotemporal dementia, type II diabetes, systemic amyloidosis, serum amyloidosis, British familial dementia, Danish familial dementia, macular degeneration and cerebrovascular amyloidosis.

71. A method according to claim 69 wherein the disease or condition is Alzheimer's Disease.

72. A method according to claim 69 wherein the tissue or fluid is cerebrospinal fluid.

73. A method for diagnosing a disease or condition in a human or animal subject, said disease or condition being characterized by the formation of amyloid deposits in neural tissue, said method comprising the step of:

A. combining tissue or fluid from a human or animal subject and a composition comprising a monoclonal antibody which binds to an epitope of a prefibrillar aggregate which forms in a human or animal contributing to an amyloid fibril formation wherein the amyloid fibril is substantially free of the epitope.

74. A method according to claim 73 wherein the disease or condition is selected from the group consisting of Alzheimer's Disease, early onset Alzheimer's Disease associated with Down's syndrome, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, sheep scrapie, and mink spongiform encephalopathy, Parkinson's disease, Huntington's disease, amyotropic lateral sclerosis, Creutzfeld Jakob disease, Gerstmann-Straussler-Scheinker syndrome, kuru, fatal familial insomnia, chronic wasting syndrome, familial amyloid polyneuropathy, frontotemporal dementia, type II diabetes, systemic amyloidosis, serum amyloidosis, British familial dementia, Danish familial dementia, macular degeneration and cerebrovascular amyloidosis.

75. A method according to claim 73 wherein the disease or condition is Alzheimers Disease.

76. A method according to claim 73 wherein the tissue or fluid is cerebrospinal fluid.

77. A diagnostic kit useful for detecting a disease or condition characterized by amyloid deposits in the central nervous system of a human or animal subject, said kit comprising:

a composition that consists of or comprises a monoclonal antibody which binds to a conformational epitope of a prefibrillar aggregate which forms in the human or animal subject and contributes to amyloid fibril formation.

78. A kit according to claim 77 wherein the monoclonal antibody is specific for a conformation-dependent epitope that is preferentially displayed by oligomeric conformations of Aβ and other amyloids.

79. A diagnostic kit useful for detecting a disease or condition characterized by amyloid deposits in the central nervous system of a human or animal subject, said kit comprising:

an isolated composition comprising a monoclonal antibody which binds to an epitope of a prefibrillar aggregate which contributes to amyloid fibril formation.

80. A kit according to claim 79 wherein the monoclonal antibody is specific for a conformation-dependent epitope that is preferentially displayed by oligomeric conformations of Aβ and other amyloids.

81. A method for treating or preventing Alzheimer's Disease and/or another amyloid disease which causes brain inflammation in a human or animal subject, said method comprising the steps of:

A) administering to the subject a therapeutically effective amount of a monoclonal antibody composition according to claim 1; and

B) administering to the subject an antiinflamatory agent in an amount that is effective to deter brain inflammation.

82. A method according to claim 81 wherein Step B comprises administering gold or a gold-containing compound to the subject in an amount that is therapeutically effective to decrease neural inflammation.

83. A method according to claim 81 wherein a colloidal gold preparation is administered in Step B.

84. A method according to claim 81 wherein the anti-inflammatory agent is combined with the monoclonal antibody.

85. A method according to claim 81 wherein the anti-inflammatory agent is separate from the monoclonal antibody.

* * * * *

Exhibit 5

In connection with Application No. 09/402,820

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Targeting Amyloid-β Peptide (Aβ) Oligomers by Passive Immunization with a Conformation-selective Monoclonal Antibody Improves Learning and Memory in Aβ Precursor Protein (APP) Transgenic Mice*[S]

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Passive immunization of murine models of Alzheimer disease amyloidosis reduces amyloid-β peptide (Aβ) levels and improves cognitive function. To specifically address the role of Aβ oligomers in learning and memory, we generated a novel monoclonal antibody, NAB61, that preferentially recognizes a conformational epitope present in dimeric, small oligomeric, and higher order Aβ structures but not full-length amyloid-β precursor protein or C-terminal amyloid-β precursor protein fragments. NAB61 also recognized a subset of brain Aβ deposits, preferentially mature senile plaques, and amyloid angiopathy. Using NAB61 as immunotherapy, we showed that aged Tg2576 transgenic mice treated with NAB61 displayed significant improvements in spatial learning and memory relative to control mice. These data implicated Aβ oligomers as a pathologic substrate for cognitive decline in Alzheimer disease.

The Aβ peptide has been hypothesized to cause the pathologic and behavioral manifestations of Alzheimer disease (AD),[3] including synaptic dysfunction and loss, neurofibrillary tangle formation, neuronal degeneration, and impaired memory. A variety of methods designed to inhibit the production or enhance the clearance of Aβ are being developed as potential AD therapies. Indeed, immunization of murine models of Aβ amyloidosis inhibits senile plaque formation and ameliorates associated cognitive impairments (1–6). Despite the development of meningoencephalitis in 6% of individuals immunized with the $A\beta_{42}$ peptide during a phase II clinical human trial (7, 8), immunotherapy, especially passive immunization, remains a compelling potential treatment for AD. Interestingly, passive immunization of mouse models of AD-like Aβ plaques has been shown to rapidly reverse learning and memory deficits without affecting Aβ plaque pathology, indicating that neutralization of toxic Aβ species can quickly restore neuronal function *in vivo* (9, 10).

The lack of learning and memory deficits in young APP transgenic mice indicates that monomeric Aβ is not responsible for behavioral impairments *in vivo* (11, 12). Furthermore, levels of soluble monomeric Aβ do not increase with age or with the onset of cognitive defects in transgenic mice (13). Therefore, if Aβ is responsible for learning and memory deficits *in vivo*, then Aβ must gain one or more of its toxic properties as a function of time. One potential mechanism for this toxic gain of function is a change in the conformation of Aβ such that it exerts its pathologic effects as an oligomeric or fibrillar macromolecule.

To specifically target toxic forms of Aβ, we developed a monoclonal antibody named NAB61 that recognizes a pathologic conformation present in Aβ dimers, soluble oligomers, and higher order species of Aβ. Using this antibody, we found that neutralization of pathologic Aβ by passive immunization of transgenic mice resulted in rapid improvement in spatial learning and memory. These results suggest that pathologic Aβ conformers produced *in vivo* are capable of disrupting neuronal function, and our data have substantiated the therapeutic potential of targeting Aβ oligomers for the treatment of AD.

MATERIALS AND METHODS

Generation of NAB61—Synthetic $A\beta_{1-40}$ (from D. Teplow, Boston, MA or from W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT) was treated with peroxynitrite, UV light, or 4-hydroxynonenal (HNE) as described (14–16). BALB/c mice were immunized with 100 μg of these Aβ species emulsified with complete Freund's adjuvant followed by three additional injections of 25 μg of Aβ emulsified with incomplete Freund's adjuvant. Isolated lymphocytes were used to generate hybridomas by fusion with Sp2/O-Ag14 myeloma cells with polyethylene glycol 1500.

Immunoprecipitation and Immunoblotting—Synthetic Aβ preparations (0.5 μg) were electrophoresed on 16% Tris-Tricine gels and immunoblotted with NAB228 or NAB61 or immunoprecipitated using NAB61 or NAB228 with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) prior to immunoblotting with NAB228. For radiolabeled APP and C-terminal APP fragments, CHO Pro5 cells transfected with pcDNA3.1 containing the cDNA for either green fluorescent protein or APP harboring the Swedish Mutation (APPswe) were radiolabeled with [^{35}S]methionine for 2 h in the presence of 200 μM MG132 (Peptides International, Louisville, KY) to enhance the accumulation of C-terminal APP fragments. RIPA buffer cell lysates

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental table and supplemental methods.

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[3] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid-β peptide; APP, amyloid-β precursor protein; APPswe, APP harboring the Swedish mutation; sAPPβ, β-cleavage-derived N-terminal ectodomain of APP; C99, β-secretase derived C-terminal APP fragment; HNE, hydroxynonenal; MCI, mild cognitive impairment; MWM, Morris water maze; RIPA, radioimmune precipitation assay; Tricine, *N*-(2-hydroxyl-1,1-bis(hydroxymethyl)ethyllglycine; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; HRP, horseradish peroxidase; Tg, transgenic.

(0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA in Tris-buffered saline, pH 8.0) containing protease inhibitors (1 μg/ml pepstatin A, leupeptin, 1-1-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone, and soybean trypsin inhibitor and 0.5 mM phenylmethylsulfonyl fluoride) were centrifuged at 100,000 × *g* for 20 min at 4 °C and immunoprecipitated with a panel of monoclonal antibodies that recognize $A\beta_{1-11}$ (NAB14, NAB61, NAB89, NAB228, Ban50), a rabbit polyclonal antibody raised against the C terminus of APP (antibody number 2493), or a goat polyclonal antibody raised against the N-terminal ectodomain of APP (Karen). NAB antibodies were generated using the oligomeric Aβ preparations as antigens but do not exhibit oligomer specificity. Immunoprecipitates were electrophoresed on 10/16.5% step gradient Tris-Tricine gels, fixed with methanol, dried, and exposed to a phosphorimaging screen for visualization. APP and APP fragments from mouse cortical extracts were detected as described (17), using the following antibodies: rabbit polyclonal antibody raised against the C terminus of APP (antibody number 5685); goat anti-N-terminal APP antibody (Karen); rabbit antisAPPβswe (antibody number 54); and NAB228. An anti-tubulin antibody (TUB2.1, Sigma) was used to detect tubulin. To detect peripheral Aβ, 200 μl of plasma was diluted with RIPA buffer and immunoprecipitated with 4G8 (anti-$A\beta_{17-24}$), which was covalently conjugated to protein A/G beads with dimethyl pimelimidate to prevent competition with endogenous IgG. Immunoprecipitates were electrophoresed on a 10/16.5% Tris-Tricine gel and immunoblotted with 4G8.

Immunocytochemistry and Immunoelectron Microscopy—Neuro2A, CHO Pro5, and NT2N neurons were transduced with a Simliki Forest Virus encoding APPswe (SFV-APPswe). After 16 h, cells were fixed with cold 95% ethanol, 5% acetic acid for 10 min followed by further permeabilization with 0.2% Triton X-100 in PBS for 10 min. Cells were stained with a goat polyclonal N-terminal APP antibody (Karen) and NAB61 followed by fluorescein isothiocyanate-conjugated anti-goat IgG and Texas Red-conjugated anti-mouse IgG. For immunoelectron microscopy, fibrillar synthetic $A\beta_{40}$ was absorbed to 300 mesh carbon-coated copper grids, washed with PBS, and blocked with 1% bovine serum albumin in PBS. Aβ fibrils were stained with NAB61 followed by anti-mouse IgG conjugated to 5-nm colloidal gold particles. Grids were then stained with 1% uranyl acetate, dried, and visualized with a Joel (Peabody, MA) 1010 transmission electron microscope.

Immunohistochemistry—Tissue blocks from human subjects or mice were immersion-fixed in 70% ethanol with 150 mM NaCl or 10% buffered formalin. Samples were dehydrated through graded ethanol solutions to xylene and infiltrated with paraffin as described (18). Sections (6 μm) were stained using standard avidin-biotin-peroxidase methods using 3-3′diaminobenzidine. Ban50 (mouse anti-$A\beta_{1-10}$), NAB228 (mouse anti-$A\beta_{1-11}$), and NAB61 (mouse anti-oligomer $A\beta_{1-11}$) were used as primary antibodies followed by HRP-conjugated anti-mouse secondary antibody (Vector, Burlingame, MD). Quantification of Aβ plaque burden in the Tg2576 mice overexpressing human APP harboring the Swedish mutation (11) was conducted as described (17). For immunofluorescence, sections were stained with a rabbit polyclonal anti-$A\beta_{42}$ antibody (BIOSOURCE International, Camarillo, CA) and with NAB61 followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG.

ELISA Analysis—For solid-phase experiments, ELISA plates (Nunc, Rochester, NY) were coated with Aβ at 1 μg/ml in PBS and blocked with 5% fetal bovine serum in PBS. Antibodies diluted in 5% fetal bovine serum/PBS were incubated at 4 °C overnight, and bound antibodies were detected with HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). For blocking experiments, ELISA plates were coated with Aβ at 0.1 μg/ml, and antibodies were preincubated with the indicated blocking peptides at 10 μg/ml. For capturing experiments, ELISA plates were coated either NAB61 or Ban50 at 10 μg/ml in PBS and blocked with 1% casein in PBS. Aβ peptides diluted in blocking buffer at 10 μg/ml were incubated at 4 °C overnight, and bound Aβ was detected with HRP-conjugated BA27 (mouse anti-$A\beta_{40}$).

For Aβ quantification, detergent-soluble fractions of cortical and hippocampal regions were obtained by sonicating samples in 1 ml of RIPA buffer containing protease inhibitors for every 150 mg of tissue. After centrifugation at 100,000 × *g* for 20 min at 4 °C, the resulting pellet was solubilized by sonication in 70% formic acid followed by another round of centrifugation to obtain detergent-insoluble Aβ. Supernatants were assayed by sandwich ELISA as described previously (19). Briefly, ELISA plates were coated with either JRF/cAβ40 or JRF/cAβ42 to capture $A\beta_{40}$ and $A\beta_{42}$, respectively. After application of diluted samples and a standard curve consisting of serially diluted synthetic Aβ (Bachem Biosciences, King of Prussia, PA), the concentration of Aβ was determined by using horseradish peroxidase-conjugated m266 (anti-$A\beta_{13-28}$) as a reporting antibody. These antibodies do not recognize the N terminus of Aβ and therefore do not compete with NAB61 for Aβ.

Immunization and Behavioral Analysis—Tg2576 mice were maintained on a C57B6/SJL F2 background by successive backcrossing to wild-type C57B6/SJL F1 females. All mice were generated and handled according to University of Pennsylvania Institutional Animal Care and Use Committee guidelines. To determine whether immunization improves learning and memory, 17–19-month-old Tg littermates were administered an initial dose of 400 μg of NAB61 or nonspecific mouse IgG (Sigma) intraperitoneally followed by maintenance doses of 200 μg as described in Fig. 5*A*. Wild-type littermates were administered either IgG or NAB61, and these two wild-type groups were analyzed as one group after treatment was determined to have no effect in non-Tg mice as described under "Results." The visible and hidden versions of the Morris water maze (MWM) were performed as described by Westerman *et al.* (12) and are described in detail in the Supplemental Methods. Briefly, mice were subjected to the visible platform MWM in which latency to reach a visibly marked platform was measured over six consecutive training blocks (four trials/block, two blocks/day). Mice were then subject to the hidden platform MWM in which the latency to reach a submerged platform was measured over 9 days (four trials/block, one block/day). Three probe trials were interspersed throughout the hidden platform MWM to test for acquisition of visual reference memory by removing the hidden platform and recording swimming behavior for 60 s. Data were recorded using a video tracking system (HVS Image, San Diego, CA). Mice were tested in a blinded manner, with groups balanced for genotype and treatment. Mice were sacrificed 3 days after the termination of the MWM for biochemical and histological assessment. Experiments were analyzed with one-way ANOVA and two-way ANOVA, with Fischer's test for post-hoc analysis. To determine whether passive immunization removed Aβ plaques, long term passive immunization was performed on a cohort of 8-month-old Tg2576 mice, which were administered weekly doses of intraperitoneal NAB61 or nonspecific IgG (500 μg) for 6 months and sacrificed at 14 months of age. Changes in Aβ deposits by immunohistochemistry and Aβ levels by ELISA were conducted as described above.

RESULTS

Generation of an Oligomer-selective Monoclonal Antibody—The role of Aβ oligomers is difficult to assess *in vivo* due to the dearth of conformation-specific molecular tools. Toward this end, we generated a monoclonal antibody that selectively recognizes oligomeric Aβ by using

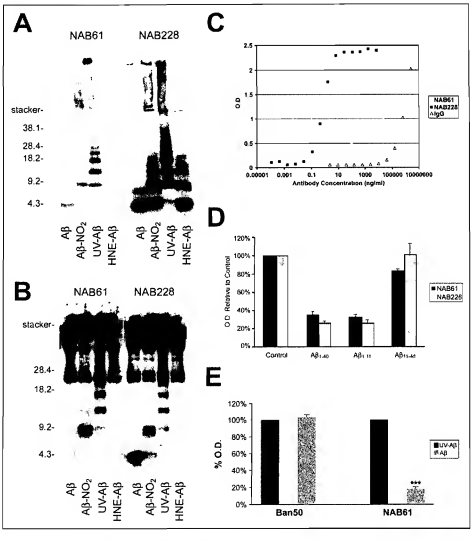

FIGURE 1. **Conformational epitope of NAB61, an Aβ oligomer-selective monoclonal antibody.** *A*, NAB61 preferentially recognizes Aβ oligomers by immunoblotting. $Aβ_{1-40}$ was treated with a peroxynitrite, UV light, or 4-HNE, electrophoresed on a 16% Tris-Tricine gel, and blotted with either NAB61 (*left*) or NAB22B (*right*). *B*, NAB61 preferentially recognizes Aβ oligomers by immunoprecipitation. $Aβ_{1-40}$ was treated with a peroxynitrite, UV light, or 4-HNE and subject to immunoprecipitation with NAB61 (*left*) or NAB22B (*right*). Immunoprecipitates were electrophoresed on a 16% Tris-Tricine gel and blotted with NAB22B. *C*, solid-phase immunoreactivity of NAB61. NAB61, NAB22B, and nonspecific mouse IgG were serially diluted and tested for immunoreactivity on ELISA plates coated with $Aβ_{1-40}$ (1 μg/ml). *D*, NAB61 recognizes an N-terminal epitope. Solid-phase ELISA immunoreactivity was blocked by preincubation of NAB61 and NAB228 with peptides corresponding to full-length Aβ (residues 1–40), the N terminus of Aβ (residues 1–11), or the C terminus of Aβ (11–40). Only peptides containing the N terminus of Aβ were able to block NAB61 and NAB228 immunoreactivity. *E*, NAB61 preferentially recognizes oligomeric Aβ when used in a sandwich ELISA as a capturing antibody. ELISA plates coated with either Ban50 or NAB61 were tested for their ability to capture Aβ or UV-cross-linked Aβ. Captured peptides were detected with HRP-conjugated 8A27 (anti-$Aβ_{35}$). A two-tailed *t* test was performed on OD readings performed in duplicate from two independent experiments (***, $p < 0.001$).

a stable oligomeric Aβ preparation as antigen. Treating synthetic $Aβ_{1-40}$ with peroxynitrite, a reactive species generated by the reaction between superoxide and nitric oxide, resulted in the formation of SDS-stable Aβ oligomers, as shown by SDS-PAGE followed by immunoblotting with NAB228, a monoclonal antibody that recognizes a linear N-terminal Aβ epitope (Fig. 1*A*). Additional stable Aβ oligomers were also generated by treating $Aβ_{1-40}$ with UV light and with the lipid-derived reactive aldehyde, 4-HNE (gifts from D. Teplow and T. Montine). Over 5,500 hybridoma supernatants were tested for the presence of Aβ antibodies, and one hybridoma was generated from a mouse immunized with nitrated Aβ that produced an IgG_1, named NAB61, with selectivity toward oligomeric Aβ species. As shown by both immunoblotting and immunoprecipitation, NAB61 showed selectivity toward SDS-stable Aβ oligomers relative to monomeric Aβ (Fig. 1, *A* and *B*, *left panels*), in contrast with other Aβ antibodies such as NAB228 (Fig. 1, *A* and *B*, *right panels*).

To further characterize this novel monoclonal antibody, we tested NAB61 in a variety of additional immunologic assays. When tested in a solid-phase ELISA format in which $Aβ_{1-40}$ was coated onto plastic, NAB61 titers were very low relative to NAB228 (Fig. 1*C*). Despite this low immunoreactivity, peptides corresponding to $Aβ_{1-11}$ and $Aβ_{1-40}$ were able to block the signal generated by NAB61, whereas $Aβ_{11-40}$ did not, indicating that NAB61 recognizes an N-terminal Aβ epitope (Fig. 1*D*). When used as a capturing antibody in a sandwich ELISA format, NAB61 had greater affinity for oligomeric Aβ relative to non-oligomeric Aβ, in contrast with other anti-Aβ monoclonal antibodies such as Ban50 (Fig. 1*E*). These *in vitro* studies indicated that NAB61 recognizes a complex conformational epitope found in the N terminus of oligomeric forms of Aβ.

Although the primary sequence of Aβ is present in full-length APP and C-terminal APP fragments, an antibody that recognizes a pathologic Aβ conformation should be specific for the Aβ peptide. Therefore, we hypothesized that NAB61 does not recognize full-length APP or C99, akin to human anti-Aβ antibodies generated upon active immunization (20). Immunoprecipitations from radiolabeled CHO cells overexpressing either green fluorescent protein or APP were performed with a panel of monoclonal antibodies that recognize the N terminus of Aβ (designated NAB antibodies), a polyclonal N-terminal APP antibody, and a polyclonal C-terminal APP antibody. All of the NAB antibodies recognized both full-length APP and C99 with the notable exception of NAB61 (Fig. 2*A*). The lack of cross-reactivity with APP was confirmed by double immunofluorescence staining of Neuro2a, NT2N, and CHO cells overexpressing APPswe, which showed that NAB61 staining did not co-localize with staining of total APP by a polyclonal N-terminal APP antibody (Fig. 2*B*). Therefore, NAB61 recognizes a conformational epitope specific to oligomeric Aβ, which is not present in the Aβ sequence when found in the context of APP or C99.

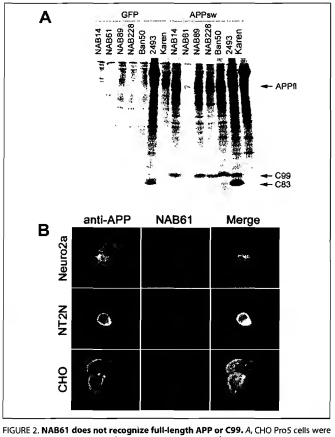

FIGURE 2. **NAB61 does not recognize full-length APP or C99.** *A*, CHO ProS cells were transfected with either green fluorescent protein (*GFP*) (*left*) or APPswe (*right*) and radiolabeled with [^{35}S]methionine for 2 h. RIPA lysates were immunoprecipitated with a panel of N-terminal monoclonal antibodies (NAB14, NAB61, NAB89, NAB228, and Ban50), a polyclonal antisera raised against the C terminus of APP, and a polyclonal antisera raised against the N-terminal ectodomain of APP. Immunoprecipitated material was electrophoresed on a 10/16.5% Tris-Tricine gel and visualized by exposure to a phosphorimaging screen. *B*, Neuro2A, NT2N neurons, and CHO ProS cells were transduced with an SFV-APPswe vector and subject to double immunofluorescence using an N-terminal APP polyclonal antibody (*Karen; green*) and NAB61 (*red*). A merged image is shown on the right with a 4′,6-diamidino-2-phenylindole counterstain for nuclei (*blue*).

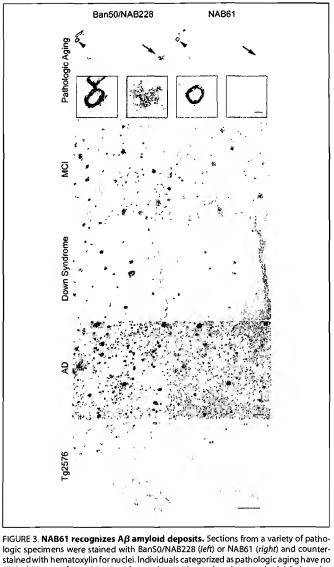

FIGURE 3. **NAB61 recognizes Aβ amyloid deposits.** Sections from a variety of pathologic specimens were stained with Ban50/NAB228 (*left*) or NAB61 (*right*) and counterstained with hematoxylin for nuclei. Individuals categorized as pathologic aging have no clinical history of cognitive impairment but do have abundant cerebral Aβ deposits. *Insets* show a higher magnification view of a blood vessel with amyloid angiopathy (*left*) or a diffuse Aβ deposit (*right*) from the mid-frontal cortex. Also shown are sections from the mid-frontal cortex of individuals with MCI, Down syndrome, and AD. Dense deposits found in brains of Tg2576 mice were also recognized by NAB61. All images were taken from serial sections with the exception of the individual with MCI.

NAB61 Immunoreactivity against Fibrillar Aβ Amyloid—To ensure that NAB61 recognized *bona fide* Aβ amyloid, immunohistochemistry was performed on a variety of tissues containing Aβ amyloid plaques. Individuals with pathologic aging (no history of cognitive impairment despite the presence of Aβ amyloid deposits), mild cognitive impairment (MCI) as demonstrated by psychometric testing, Down syndrome, and AD all contained Aβ plaques and other Aβ deposits that were NAB61-immunoreactive (Fig. 3). Interestingly, diffuse amyloid plaques were poorly stained by NAB61, despite robust staining of amyloid angiopathy (see Fig. 3, *insets*, for pathologic aging). Compact amyloid plaques from Tg2576 transgenic mice overexpressing APPswe were also recognized by NAB61 (Fig. 3).

The initial stages of AD pathology are generally characterized by Aβ amyloid pathology in association cortices such as the mid-frontal cortex. With disease progression, Aβ amyloid in neocortical regions becomes more advanced, co-incident with the development of relatively milder Aβ deposits in limbic regions such as the entorhinal cortex and the hippocampus (21). NAB61 immunoreactivity generally exhibited regional selectivity, which reflected the regional progression and severity of Aβ amyloid pathology. For example, many diffuse amyloid deposits in the hippocampus and entorhinal cortex of AD brains were poorly stained by NAB61 (Fig. 4*A*, *left* and *middle panels*) despite strong staining of mature senile plaques and amyloid angiopathy (Fig. 4*A*, *arrowheads*, and 4*B*), whereas many Aβ amyloid plaques in the mid-frontal cortex showed more robust NAB61 immunoreactivity (Fig. 4*A*, *right panels*).

The regional selectivity of NAB61 immunoreactivity suggested that NAB61 recognizes a conformation that is found in advanced, pathologic Aβ deposits, namely mature senile plaques and amyloid angiopathy. These inclusions are distinct from diffuse Aβ deposits, which are not associated with neuritic alterations, tau pathologies, or neuronal loss. Double immunofluorescence staining showed that many amorphous Aβ deposits, which were recognized by a conventional anti-Aβ42 antibody, were not recognized by NAB61 (Fig. 4*B*). In contrast, mature senile plaques were labeled by both antibodies (Fig. 4*B*). Given that Aβ fibrils are the ultrastructural building blocks of senile plaques, NAB61 staining of synthetic Aβ fibrils by immunoelectron microscopy further corroborated the ability of NAB61 to recognize pathologic forms of Aβ (Fig. 4*B*). Therefore, NAB61 appears to recognize a pathologic confor-

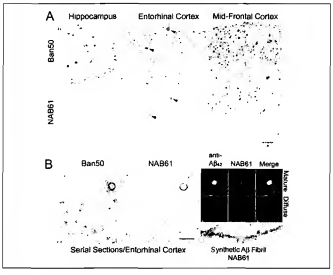

FIGURE 4. ***NAB61 recognizes a subset of fibrillar Aβ pathology.*** *A*, regional distribution of NAB61 immunoreactivity. Sections from the hippocampus, entorhinal cortex, and mid-frontal cortex from individuals with AD display different amounts of NAB61 immunoreactivity. In cases of AD in which limbic regions contain predominantly diffuse-type Aβ deposits, NAB61 immunoreactivity was not abundant, although amyloid angiopathy and dense senile plaques were strongly stained (*arrowheads*). In contrast, abundant staining was observed in regions such as the mid-frontal cortex, where dense, mature senile plaques predominate. *B*, a higher magnification view of serial sections from the entorhinal cortex of an individual with AD clearly demonstrates that NAB61 does not recognize all Aβ deposits (*left panels*). Double immunofluorescence with a polyclonal anti-$Aβ_{42}$ antibody (*green*) and NAB61 (*red*) of the entorhinal cortex demonstrates that NAB61 recognizes a mature, dense, cored senile plaque. In contrast, a diffuse Aβ deposit was not stained by NAB61 (*upper right panels*). NAB61 recognizes Aβ fibrils by immunoelectron microscopy. NAB61 staining of synthetic $Aβ_{1-40}$ fibrils was detected with 5 nm colloidal gold-conjugated anti-mouse IgG (*lower right panel*).

mation present in dimeric and oligomeric Aβ, which is maintained during fibrillization and coalescence into senile plaques. NAB61 did not recognize other inclusions consisting of amyloidogenic proteins such as neurofibrillary tangles or Lewy bodies (data not shown), indicating that NAB61 is specific for Aβ and does not recognize a pathologic conformation common to other amyloidogenic proteins.

NAB61 Improves Spatial Learning and Memory—Learning and memory impairments have been shown to normalize rapidly upon neutralization of Aβ following passive immunization of murine models of Aβ amyloidosis (9, 10). To probe the role of Aβ oligomers on cognitive dysfunction, 17–19-month-old Tg2576 mice were immunized with NAB61 (n = 14) or nonspecific IgG (n = 16) and tested in the MWM for spatial learning and memory, using the schedule shown in Fig. 5*A*. Non-transgenic mice were also treated with either IgG (n = 7) or NAB61 (n = 7). However, for statistical analysis, data from IgG- and NAB61-treated non-transgenic control mice were pooled after performing a two-way ANOVA, which revealed no effect of treatment on performance in the hidden water maze for non-Tg mice (treatment, p = 0.8974; block, p = 0.0251; interaction, p = 0.8942).

To test for potentially confounding sensorimotor or motivational defects, latencies to reach a visible platform were measured over successive training blocks (four trials/block). Non-transgenic, NAB61-treated Tg2576, and IgG-treated Tg2576 mice showed no deficits in the visible water maze (Fig. 5*B*). Although latencies for both NAB61-treated and IgG-treated Tg2576 mice on the second and third training blocks of the visible water maze tended to be higher than latencies for non-transgenic mice, differences in overall performance were insignificant (repeated measures ANOVA p = 0.0814). Furthermore, the latency to reach the visible platform and swim speeds on both the first trial and the first block were not statistically different between the three groups (data not shown), arguing against the presence of confounding sensorimotor or motivational deficits.

Spatial learning and memory were then tested using the hidden water maze in which the primary measure of learning and memory was latency to reach the hidden platform (Fig. 5*C*). IgG-treated Tg2576 mice showed no significant improvement in latencies over the testing period (one-way ANOVA, p = 0.4402), whereas NAB61-treated Tg2576 mice and non-transgenic mice both showed a significant decrease in latency with training (one-way ANOVA: NAB61, p = 0.0004; non-transgenic, p = 0.0184). Furthermore, comparisons between the three groups of mice demonstrated that both non-transgenic and NAB61-treated Tg2576 mice performed significantly better than IgG-treated Tg2576 mice (repeated measures ANOVA, p = 0.0002; non-transgenic *versus* IgG, p = 0.0014; NAB61 *versus* IgG p = 0.0006). Therefore, passive immunization with NAB61 ameliorates behavioral deficits in the hidden water maze.

To confirm that the improved behavior on the hidden water maze was due to the acquisition of spatial reference memory, three probe trials were interpolated throughout the training process (Fig. 5*A*) in which the platform was removed, and the percentage of time spent searching in the target quadrant (where the platform is usually located) was determined. During the first probe trial, the three groups of mice exhibited spatially oriented swimming behavior, indicating that all three groups have acquired some degree of a spatial reference for the general location of the hidden platform (Fig. 5*D*). However, the time spent in the target quadrant relative to adjacent quadrants was only significantly different for NAB61-treated Tg2576 and non-transgenic mice. After further training, this behavioral measure became saturated and thus was unable to discern any differences between the three groups of mice in the final two probe trials (Fig. 5, *E* and *F*).

Since the time spent in the target quadrant appeared to plateau by the second of the three probe trials, we used a third measure of spatial learning to confirm the improved acquisition of spatial reference memory upon NAB61 immunization. A platform crossing index was calculated that measures the number of crossings over the exact location of the platform subtracted by the average number of crossings over the platform locations in non-target quadrants (Fig. 5*G*). Using this measure, both NAB61-treated Tg2576 mice and non-transgenic mice performed significantly better than IgG-treated Tg2576 mice (repeated measures ANOVA, p = 0.0301; NAB61 *versus* IgG, p = 0.0332; non-transgenic *versus* IgG, p = 0.0426). Again, this effect was not due to the presence of motor deficits as all three groups of mice exhibited similar swim speeds regardless of the probe trial (Fig. 5*H*, two-way ANOVA: group, p = 0.4033; probe trial, p = 0.8911; interaction, p = 0.9804). Therefore, three statistical measures (latency, percentage of time in target quadrant, and platform crossing index) all indicated that short term immunization with NAB61 improved spatial learning and memory in aged Tg2576 mice.

NAB61 Immunization Does Not Affect APP Processing or Aβ Accumulation—To show that the NAB61-mediated neutralization of Aβ oligomers was independent of effects on APP processing or the extent of Aβ amyloid pathology, we examined levels of APP and APP fragments in mice after NAB61 treatment. Steady-state levels of full-length APP, sAPPβ, and C99 were not different among IgG-treated and NAB61-treated Tg2576 mice, arguing that NAB61 did not affect proteolytic processing of APP (Fig. 6*A*). Furthermore, no differences in amyloid plaque morphology, distribution, or density were noted upon NAB228 or NAB61 immunohistochemistry (Fig. 6*B*). Additionally, quantification of detergent-soluble and insoluble levels of cortical and hippocampal Aβ by sandwich ELISA indicated that Aβ levels were not statistically different upon short term NAB61 immunization (Fig. 6*C*). Surprisingly, we also found that 6 months of peripheral passive immu-

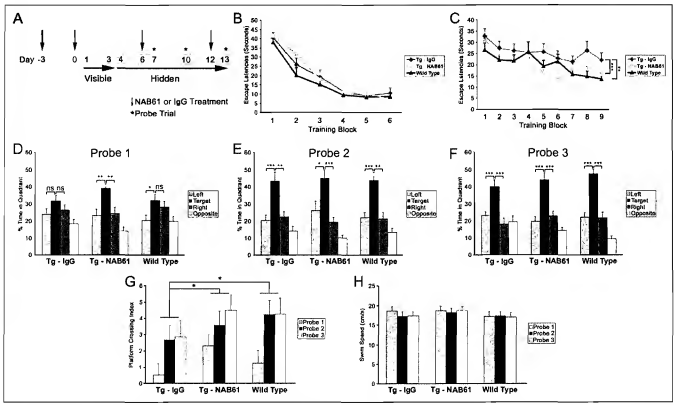

FIGURE 5. **NAB61 improves spatial learning and memory.** *A*, immunization and behavioral testing. 17–19-month-old Tg2576 mice were administered an initial dose of 400 μg of NAB61 or nonspecific mouse IgG on day −3 with maintenance doses of 200 μg on days 0, 6, and 12. Performance on the visible water maze was tested on days 1–3 (four trials/block, two blocks/day) followed by the hidden water maze on days 4–13 (four trials/block, one block/day). Probe trials were interpolated throughout the hidden water maze on days 7, 10, and 13. *B*, performance in the visible water maze. NAB61-treated Tg2576, IgG-treated Tg2576, and non-transgenic mice all learned the visible water maze task. No significant differences were found between the three groups. *C*, performance in the hidden water maze. Within-group analysis indicated that NAB61-treated Tg2576 and non-transgenic mice both showed significant improvements in escape latencies with time, whereas data from IgG-treated Tg2576 mice did not reach statistical significance. Between-group analysis indicated that both NAB61-treated Tg2576 and non-transgenic mice both performed significantly better than IgG-treated Tg2576 mice. (**, $p < 0.01$; ***, $p < 0.001$). *D–F*, spatial reference memory, percentage of time in quadrant. The percentage of time spent in each quadrant for a 60-s probe trial conducted early (*D*), middle (*E*), and late (*F*) during training indicated that all three groups of mice acquired some level of spatial reference memory. However, only NAB61-treated Tg2576 and non-transgenic mice spent significantly more time in the target quadrant relative to adjacent quadrants during the first probe trial (*D*). *ns*, not significant; $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *G*, spatial reference memory, platform crossing index. The number of crosses over the exact location of the hidden platform subtracted by the average number of crosses over non-target platforms was measured throughout the three probe trials. NAB61-treated Tg2576 and non-transgenic mice performed significantly better than IgG-treated Tg2576 mice. (*, $p < 0.05$). *H*, motor function in the Morris water maze. All three groups of mice displayed similar swim speeds in all three probe trials.

nization (Table 1) or direct intracerebral inoculation (supplemental Table 1) performed in additional cohorts of Tg2576 mice did not reduce Aβ amyloid burden, further arguing that the effect of NAB61 on cognitive behavior was via direct neutralization of Aβ oligomers and not on APP processing or Aβ amyloid pathology.

Finally, passive immunization against Aβ has been postulated to enhance the efflux of Aβ from the central nervous system into the periphery. However, the presence of Aβ oligomers has not been demonstrated in the periphery, and therefore, NAB61 would not be expected to affect peripheral pools of Aβ. Immunoprecipitation of Aβ from sera obtained from passively immunized Tg2576 mice failed to demonstrate an increase in peripheral Aβ (Fig. 6*D*). Furthermore, naturally occurring Aβ oligomers were not immunoprecipitated from sera samples, although very low concentrations of synthetic Aβ oligomers could be immunoprecipitated when added to sera samples (Fig. 6*D*). Having ruled out effects on APP processing, Aβ amyloid accumulation, and peripheral pools of Aβ, the selectivity toward oligomeric Aβ exhibited by NAB61 suggested that direct neutralization of Aβ oligomers by immunization with NAB61 can reverse learning and memory deficits in Tg2576 mice.

DISCUSSION

Despite the use of a stable Aβ oligomer preparation, the generation of an anti-Aβ oligomer-selective monoclonal antibody was a low probability event, with less than 0.02% of tested hybridomas generating an antibody with selectivity toward Aβ oligomers. Indeed, although several monoclonal antibodies that recognize Aβ or nitrated epitopes were also isolated, none of these antibodies showed any Aβ oligomer selectivity. Given its unique epitope, we have thoroughly characterized NAB61 using multiple methods including immunoblotting, immunoprecipitation, solid-phase ELISA, sandwich ELISA, immunocytochemistry, immunohistochemistry, immunoelectron microscopy, and immunotherapy. In contrast with other reported oligomer- or amyloid fibril-specific antibodies (22, 23), NAB61 does not recognize other amyloidogenic proteins and is specific for Aβ, recognizing both oligomers and higher order Aβ structures. Thus, NAB61 recognized a pathologic Aβ conformation present early in the process of oligomerization, which is maintained even in Aβ fibrils. Additional polyclonal anti-Aβ sera have been reported that are apparently similar to NAB61 by immunoblotting. However, only one of the antibodies has been characterized by immunoprecipitation, immunohistochemistry, and immunoelectron microscopy (24–26). Furthermore, NAB61 is a monoclonal antibody, and therefore, suitable for passive immunization and for future development as a therapy for AD.

The occurrence of aseptic meningoencephalitis in 6% of individuals immunized with $A\beta_{42}$ halted a phase II human clinical trial and calls into question the safety of active immunization for the treatment of AD

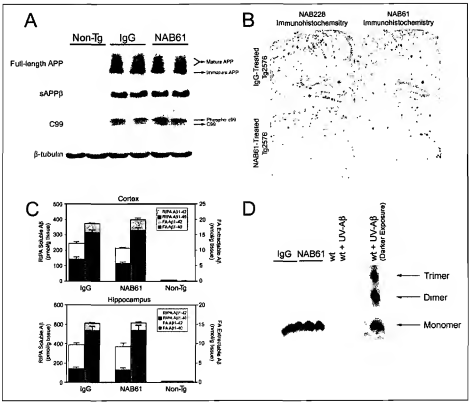

FIGURE 6. **Sub-chronic NAB61 treatment does not affect APP or Aβ levels.** *A*, APP processing in NAB61 immunized mice. Full-length APP was immunoprecipitated with a C-terminal APP antibody (56B5) and immunoblotted with an N-terminal APP antibody (Karen). sAPPβswe levels were assessed by immunoblotting with an end-specific polyclonal antibody (54). C99 was immunoprecipitated with NAB22B, run on a 10/16.5% Tris-Tricine gel, and immunoblotted with 56B5. β-tubulin was immunoblotted with TUB2.1. *B*, Aβ amyloid pathology in NAB61 immunized mice. Serial sections from Tg2576 mice treated with either non-specific IgG (*top*) or NAB61 (*bottom*) were subject to immunohistochemistry using either NAB22B (*left*) or NAB61 (*right*) as primary antibodies, showing that short term NAB61 immunization did not clear amyloid deposits. *C*, quantification of Aβ accumulation in NAB61 immunized mice. Cortical and hippocampal regions were subject to sequential extraction with RIPA and then 70% formic acid (*FA*). Aβ concentrations were measured by sandwich ELISA specific for $Aβ_{1-40}$ and $Aβ_{1-42}$. The short NAB61 immunization protocol did not alter levels of Aβ accumulation. *D*, peripheral pools of Aβ in NAB61 immunized mice. Plasma samples from Tg2576 mice treated with either nonspecific IgG or NAB61 were subject to immunoprecipitation and immunoblotting with 4G8, demonstrating that NAB61 immunization does not result in the peripheral accumulation of Aβ. Although Aβ oligomers (1 ng of untreated Aβ and 1 ng of UV-cross-linked Aβ) could be detected when spiked into plasma from non-transgenic mice, no Aβ oligomers could be detected in plasma from Tg2576 mice. *wt*, wild type.

TABLE 1

Aβ quantification of Tg2576 mice immunized with NAB61

Tg2576 mice were treated with weekly intraperitoneal injections of 500 μg of non-specific IgG ($n = 8$) or NAB61 ($n = 9$) from 8 to 14 months. Detergent-soluble and insoluble fractions were obtained from the cortex and hippocampus (Hipp) of treated mice by sequential extraction with RIPA buffer and formic acid.

	Aβ40			Aβ42		
	IgG[a]	NAB61[a]	*p* value[b]	IgG[a]	NAB61[a]	*p* value[b]
	pmol/g			*pmol/g*		
RIPA						
Cortex	25.3 ± 1.5	23.9 ± 0.9	0.43	6.9 ± 1.5	4.2 ± 0.5	0.08
Hipp	24.4 ± 1.0	22.8 ± 0.8	0.22	3.3 ± 0.2	2.8 ± 0.2	0.14
FA						
Cortex	2830.7 ± 529.7	3170.8 ± 538.1	0.66	655.1 ± 94.3	864.6 ± 164.2	0.30
Hipp	1449.5 ± 221.8	1039.5 ± 172.2	0.16	449.6 ± 55.3	360.8 ± 60.0	0.30

[a] Aβ levels were quantified using JRF-m266 sandwich ELISA, and are presented as average values ± standard error.
[b] Statistical analysis using two-tailed t-tests showed no significant differences between treatment groups.

(7, 8). Treatment using anti-Aβ antibodies has been proposed as a safer alternative to active immunization since immunotherapy-induced meningoencephalitis appears to be due to the activation of autoreactive T cells and does not correlate with the presence of anti-Aβ antibodies (8). Furthermore, Aβ oligomer-selective antibodies are decreased in AD patients, suggesting that replacement therapy with such antibodies may be appropriate (27). *A priori*, the selectivity of NAB61 for pathologic forms of Aβ, in addition to the lack of cross-reactivity with APP or C99, indicated that NAB61 may be a safer alternative for therapy when compared with other monoclonal anti-Aβ antibodies. However, we have reported one case of meningoencephalitis after peripheral immunization of a 19-month-old Tg2576 mouse with NAB61 (28). This isolated case was not part of the behavioral cohort presented here, and the mechanism triggering Aβ vaccine-related meningoencephalitis is still unknown. Nonetheless, current passive immunization trials should continue with careful regard toward unwanted complications. Additionally, the low titer of NAB61 by solid-phase ELISA indicated that the measurement of antibody response in actively immunized individuals by ELISA methods may lead to false negative results.

Although we have shown that NAB61 treatment improves spatial learning and memory in 17–19-month-old Tg2576 mice through the measurement of three different behavioral indices, NAB61 has been ineffective thus far in clearing Aβ pathology even in Tg2576 mice treated for 6 months. The successful clearance of amyloid pathology by passive immunization has been reported in PDAPP mice and very old (>16 months) Tg2576 mice (2, 29–32), both of which are characterized by the presence of considerable amounts of diffuse Aβ. However, our long term passive immunization trial was performed in younger Tg2576 mice (<14 months) in which compact amyloid deposits predominate, perhaps accounting for the lack of effect on the burden of Aβ pathology following the schedule used here. Alternatively, the Aβ oligomers recognized by NAB61 may not be easily cleared by immune-mediated mechanisms. NAB61 is also an IgG_1 that has been shown to be less effective in reducing Aβ amyloid burden in transgenic mice (33).

Indeed, distinct morphological subtypes of Aβ amyloid plaques are differentially cleared depending on the anti-Aβ antibody isotype (34). Thus, NAB61 may exert its behavioral effect by blocking the biological activity of pathologic Aβ oligomers, and at the same time, be ineffective in promoting the immune-mediated clearance of Aβ.

Finally, soluble oligomeric forms of Aβ have been postulated to contribute to the onset of AD, and they may affect neuronal function initially by impairing synaptic function (35–39). Furthermore, although insoluble amyloid plaques are found very early in the disease process in patients with early AD or mild cognitive impairment, soluble Aβ levels are also increased in these individuals, and soluble Aβ levels correlate better with neurofibrillary degeneration and the loss of synaptic markers than do amyloid plaques (40–42). Similarly, decreases in synaptophysin immunoreactivity and impairments in synaptic transmission in APP transgenic mice precede the onset of microscopic Aβ amyloid pathology (43, 44). With mounting evidence for the synaptotoxic effects of soluble Aβ oligomers, our study corroborates previous reports indicating that immunization can ameliorate cognitive defects independent of APP processing and levels of insoluble Aβ (9, 10). Furthermore, since NAB61 recognizes a conformation found on oligomeric forms of Aβ, we have provided strong evidence that Aβ oligomers generated *in situ* disrupt neuronal function. Thus, we propose that targeting the pathologic conformation recognized by NAB61 may be useful in the treatment of AD and that further elucidation of the conformation recognized by NAB61 may yield insights into the mechanisms underlying the synaptotoxic effects of Aβ as well as assist in determining the roles of different conformational pools of Aβ to the development of dementia.

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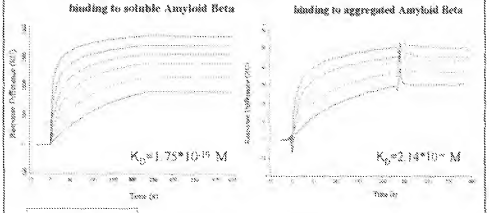
Exhibit 6

In connection with Application No. 09/402,820

Exhibit 6A: Antibody "A" - Affinity to Soluble and Aggregated Aβ - BIAcore Analysis



Antibody A has a 10-fold higher affinity to soluble Aβ 1-40

Exhibit 6A: Antibody "A" - Competition ELISA on Soluble vs Aggregated Aβ 1-40

Antibody A seems to have a 10-fold binding preference to soluble Aβ as compared to aggregated Aβ

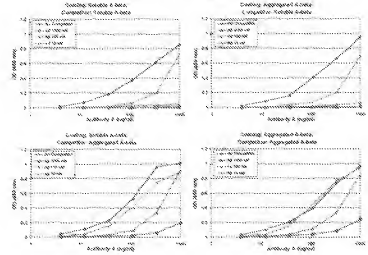
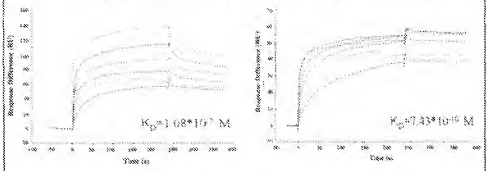


Exhibit 6B: Antibody "B" - Affinity to Soluble and Aggregated Aβ: BIAcore Analysis



Antibody B has ~ 100-fold higher affinity to aggregated Aβ 1-40

Exhibit 6B: Antibody "B" - Competition ELISA on Soluble Vs Aggregated Aβ 1-40

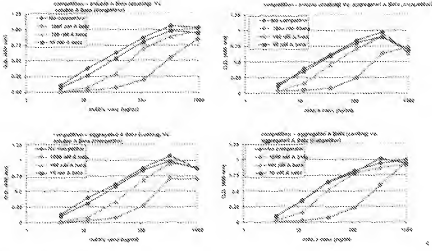


Exhibit 7

In connection with Application No. 09/402,820

Exhibit 7

Western Blot Analysis of Soluble and Aggregated Aβ 1-40

Soluble (S) and aggregated (A) Aβ 1-40 were resolved on SDS-PAGE gel, blotted onto a nitrocellulose membrane, and probed with either Antibody A or Antibody B.